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**ECOLOGY AND CONTROL OF**  
***FUSARIUM* SPECIES AND**  
**MYCOTOXINS IN WHEAT GRAIN**

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## ABSTRACT

This study investigated the effect of interacting environmental factors on the ecophysiology of *Fusarium culmorum* and *F. graminearum* and mycotoxin production, and potential for controlling growth and deoxynivalenol (DON) and nivalenol (NIV) production using antioxidants and essential oils on wheat grain. Two-dimensional temporal profiles of growth and DON/NIV production for *F. culmorum* and *F. graminearum* were constructed for the first time and showed that the water activity ( $a_w$ ) ranges required for growth and DON/NIV production were different *in vitro* and on wheat grain. Growth occurred over a wider  $a_w$  range (0.9-0.99) while DON/NIV production occurred over a significantly narrower range (0.95 – 0.99  $a_w$ ) at both 15 and 25°C over a 40 day incubation period. Interactions between  $a_w$ , temperature, and sub-optimal concentrations of fungicides had a significant impact on growth and competitiveness of *F. culmorum* in interspecific interactions with 5-6 other wheat grain fungi. This had an impact on both colonisation capacity *in vitro* and *in situ* and affected DON/NIV production. Interactions were complex changing with environmental or fungicide stress resulting in significant stimulation or reduction of both fungal growth and mycotoxin production. An Index of Dominance was developed to compare interspecific interactions between *F. culmorum* and other wheat spoilage fungi and showed that competitiveness of *F. culmorum* increased as temperature and  $a_w$  level approached the optimum conditions for growth and DON/NIV production. Niche overlap indices (NOI) and niche maps were developed for the first time and showed that interspecific competitiveness was closely related to the number of nutritional carbon sources shared between spoilage fungi. Hydrolytic enzyme profiles for seven different enzymes by *F. culmorum*, other *Fusaria* and spoilage fungi showed that the most competitive species produced the highest specific activity of N-acetyl- $\beta$ -D-glucosaminidase. Potential for control of *Fusarium* species post-harvest using 23 essential oils and 8 antioxidants was investigated. Cinnamon, clove, bay, butylated hydroxyanisole and propyl paraben (500  $\mu\text{g g}^{-1}$ ) were able to significantly reduce (>90%) the colonisation of wheat grain by three *Fusarium* species and DON/NIV production. Bay essential oil was the most effective at controlling total fungal populations and DON/NIV in naturally contaminated wheat grain.

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# **CHAPTER 1**

## **LITERATURE REVIEW AND OBJECTIVES**



## 1.1 GENERAL OVERVIEW

*Fusarium* ear blight (FEB) is a cereal disease responsible for significant reduction in yield and quality of wheat grain throughout the world. FEB is a pre-harvest disease, but *Fusarium* species can grow post-harvest if wet grain is not dried efficiently and quickly. In addition to the degradation in grain quality, *Fusarium* species produce an array of mycotoxins which contaminate the grain (Parry *et al.*, 1995; Jennings *et al.*, 2000; Magan *et al.*, 2002; Bottalico & Perrone, 2002). These mycotoxins are harmful to both animals and humans and cause a wide range of different symptoms of varying severity.

*Fusarium* is currently controlled pre- and post-harvest by the application of fungicides and effective storage regimes. However, some strains of *Fusarium* are resistant to fungicides and therefore new fungicidal agents are required (D'Mello *et al.*, 1999; Placinta *et al.*, 1999; Khan *et al.*, 2001). An additional complication when controlling *Fusarium* is that mycotoxin production may be stimulated by fungicidal action, especially when fungicides are present in sub-optimal concentrations (D'Mello *et al.*, 1998; Matthies *et al.*, 1999; Matthies & Buchenauer, 2000; Jennings *et al.*, 2000; Hope *et al.*, 2000).

Environmental conditions have been shown to affect fungal colonisation, mycotoxin production and fungicide efficiency. However, very few studies consider key environmental factors such as available water and temperature. Results generated under limited conditions are not accurate models for what occurs in the field. To enable a more complete "picture" to be represented in the screening and in-vitro experiments, multi-target environmental technological approaches should be adopted. The interaction between the pathogen and other fungi, which inhabit the same niche, is also rarely considered. This is short sighted as the interaction between *Fusarium*, its environment and interspecific interactions with other fungi have been shown to be significant (Lee & Magan, 1999a; Lee & Magan, 1999b).

This study investigated how *Fusarium culmorum* isolates from northern Europe are affected by available water, temperature, fungicidal agents and other fungi that inhabit

the same ecological niche. The combined effects of these different variables were also investigated. In addition to how the morphology and ecology of the species is affected by environmental conditions, the effect on mycotoxin production (in particular deoxynivalenol) was also determined. Potential for using antioxidants and essential oils to control *Fusarium* and deoxynivalenol / nivalenol in mouldy grain was also considered.

## **1.2 WATER AVAILABILITY**

Fungi require water to grow. Water is present in different percentages in substrates. The total water content of a substrate can be expressed as percentage moisture content. This is a ratio between dry weight and wet weight, which may be expressed in terms of either dry or wet weight. However, percentage moisture content does not indicate the quantity of water that is readily available to an organism for metabolism. This is because water exists in different states within a substrate.

Basically water is found in two main states: Constitutive water, which is chemically bound to the substrate molecules, and free water which is either absorbed or adsorbed to the substrate (Pixton, 1976; Aldred, 2000). It is the free water present in the substrate that has the largest effect on microbial growth as it is available to the organism with little or no "metabolic work". Water availability can be expressed as water activity ( $a_w$ ) or as water potential ( $\psi$ ).

### **1.2.1 Water activity**

Water activity is the ratio of the vapour pressure of the water in a substrate to that of pure water at the same temperature and pressure (Ayerst, 1965). Labuza (1974) linked  $a_w$  to equilibrium relative humidity with the following expression:-



$$a_w = \frac{p}{p_o} = \frac{\%ERH}{100}$$

Where;

p	= vapour pressure of water in solution or solid substrate
p <sub>o</sub>	= vapour pressure of pure water at experimental temperature and pressure.
ERH (%)	= equilibrium relative humidity at which solution or solid substrate neither gains nor loses moisture to the atmosphere.

This equation produces a number in the range 0-1.0 which represents the ability of the water present to humidify the immediate environment, with a reference pool of pure water being 1.0. Temperature is important when considering  $a_w$ . This is because at higher temperatures samples evaporate faster and vapour pressure is increased.  $A_w$  will therefore increase with increasing temperature.  $A_w$  is used in preference to moisture content when working with microorganisms as it gives a direct measure of the proportion of water in the sample that is available for growth and metabolism (Aldred, 2000).

### 1.2.2 Water potential

$A_w$  does not allow the constraints of a system to be separated into its component parts (i.e osmotic, matric and turgor potentials). These components are of particular interest to studies involving soils or other complex substrate matrices. Water potential does allow for the components to be separated. Magan and Lacey (1988) defined water potential as the potential free energy in a system relative to a hypothetical pool of pure free water of specific mass. The energy in this system is measured in Pascals (Pa). Pure water in this method is assigned a value of zero. Thus chemically or physically constrained water has a negative value and requires physiological action on

the part of the organism to make the water available by raising the thermodynamic potential. Water potential is related to  $a_w$  with the following equation:

$$\psi = P \left( \frac{RT}{V_w} \right) \ln a_w$$

Where;

- P = Pressure
- R = Gas constant
- T = Absolute temperature (K)
- $V_w$  = potential modal volume of water.

### 1.2.3 Detection methods

A number of instrumental methods may be used to determine the water availability of a material. An electronic hydrometer may be used to calculate ERH and therefore  $a_w$ . This type of detector requires frequent recalibration with standard saturated salt solution and was prone to the sensor becoming damaged at high  $a_w$  (Kitu, 1985). However, in modern instruments these problems have been lessened by more efficient technology.

Psychometric measurement may also be used to determine  $a_w$ . In this technique a thermocouple is cooled to below the dew point in the headspace humidified by the sample. The rate of evaporation from the thermocouple is measured and therefore the vapour pressure of the headspace can be determined.

$A_w$  measurement instruments are all impeded by the time it takes for the sample to equilibrate within the sample chamber. This has been alleviated by mechanically mixing the headspace of the chamber in some instruments. However, this causes problems with fine particulate samples such as flour.



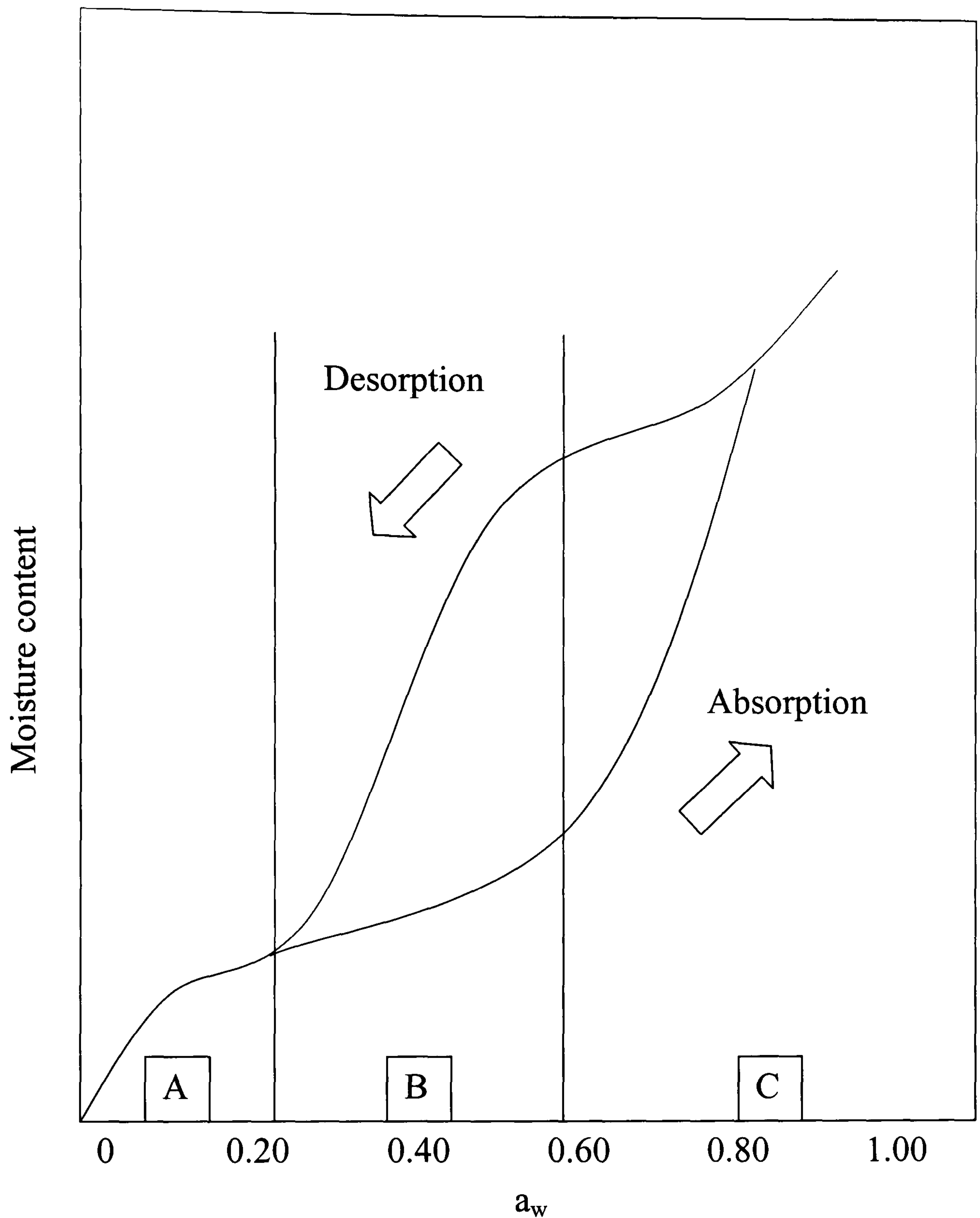
### 1.2.4 Moisture sorption isotherms

Moisture content may be related to  $a_w$  by the use of moisture sorption isotherms. This is necessary if you wish to adjust  $a_w$  of a solid substrate by manipulating the moisture content (for instance drying grain). The sigmoidal curve produced is specific for the substrate and the condition. It is also altered by the hysteresis effect caused by the addition or removal of water to adjust the  $a_w$  (Figure 1.1). The data points for the curve are normally determined experimentally as mathematical models are complex and do not encompass all material types (Chen & Morey, 1989).

### 1.2.5 Effect of $a_w$ on fungal growth

Fungal growth has been shown to occur in an  $a_w$  range of 1.0 to 0.6 for extreme xerophiles (Troller & Christian, 1978). If grown at sub-optimal  $a_w$  levels growth rates are reduced and lag times increased. Fungi will tolerate the widest range of conditions at optimal  $a_w$  and at optimal growth conditions the largest range of  $a_w$  is supported. Therefore, as  $a_w$  deviates from the optimum for any given fungus, so the range of environmental conditions it may grow under is also reduced. Magan and Lacey (1988) found that pH, temperature and gas balance stress interacted with lowered  $a_w$  to markedly inhibit germination and growth.

Fungi are not defenceless against  $a_w$  changes and may employ a number of systems to combat low  $a_w$  conditions. For instance, membrane permeability may be changed to promote selective ion transport, followed by the biosynthesis of "compatible" solutes (mainly polyols such as glycerol and mannitol). This process causes the water potential of the cytoplasm to decrease, facilitating water uptake by the cell (Peppendick & Mulla, 1985).



**Figure 1.1** Moisture Isotherm showing hysteresis effect (Labuza, 1968). A, monolayer formation. B, additional layers added to monolayer. C, condensation in pores, capillary effects and dissolution of soluble components.



The water availability "stress", although found to inhibit growth has also been shown to enhance conidiation (Cahagnier, 1993). Conidiation is a secondary process caused by differentiation response and is associated with secondary metabolite production and thus mycotoxins, as they are secondary metabolites. Therefore it is important to understand how water stress and fungicide interactions affect fungal growth and mycotoxin production.

### 1.3 MYCOTOXIGENIC GRAIN SPOILAGE FUNGI

Fungi that produce mycotoxins on cereals can be roughly categorised into two groups; field and storage. Generally field fungi are considered pathogenic and storage species saprophytic (Jenkins *et al.*, 1988). However, it should be noted that the origin of both is the field. Invasion pre-harvest is mostly reliant on fungi/host and other organism interactions. Post-harvest, the following are important influences on fungal establishment:-

- Nutrients (i.e Grain type and quality)
- Physical environment (i.e temperature,  $a_w$ )
- Biotic factors (i.e insect, plant, fungal interactions)

Toxigenic fungi can be separated further into four different types:

- Plant pathogens (e.g. *F. culmorum*).
- Fungi that grow and produce mycotoxins on senescent or stressed plants. (e.g. *F. verticillioides*).
- Fungi that initially colonise a plant and predispose the commodity to mycotoxin contamination post-harvest. (e.g. *Aspergillus flavus* - not in tropics).
- Fungi found in the soil or on decaying plant material that infects the developing kernels in the field and later proliferates in storage. (e.g. *A. ochraceus*).

### 1.3.1 *Fusarium* species

The genus *Fusarium* contains some of the most important plant pathogenic species of fungi (Placinta *et al.*, 1999; Abbas *et al.*, 2000; Cooney *et al.*, 2001). *Fusarium* species cause disease in many economically important crops. The diseases caused by various species of *Fusarium* are:- wilts, blights, root rots, and cankers in legumes, coffee, pine trees, wheat, corn, carnations and grasses. *Fusarium* may infect crops both pre- and post-harvest. Many *Fusarium* species also produce mycotoxins (Table 1.1), which are dangerous to humans and animals. For example, the T-2 mycotoxin was found to be responsible for the death of 100,000 Russians between 1942 and 1948 when grain contaminated with *Fusarium sporotrichioides* was not properly dried over the winter and then consumed. The T-2 toxin causes alimentary toxic aleukia (ATA). Outbreaks of ATA also occurred in Russia between 1913 and 1932 (Jacobsen *et al.*, 1993).

### 1.3.2 Taxonomy

The Taxonomy of *Fusarium* was in disarray until an international collaborative group (Nelson *et al.*, 1983) reorganized the taxonomy of the genus. Thirty species were identified within the genus *Fusarium* at this time. Due to the taxonomic problems some *Fusarium* species were incorrectly identified as being responsible for the production of certain mycotoxins. For instance *M. nivale* and *M. nivale* var. *majus* were previously considered to be within the genus *Fusarium*. Unlike *F. culmorum*, *M. nivale* and *M. nivale* var. *majus* do not form chlamydospores in soil, instead surviving in a growing state on organic matter in the soil. These fungi prefer colder conditions than *Fusarium* species and cause the disease Snow Mould, which infects the leaves of barley. They may also infect wheat ears like *Fusarium* species either homogenously or combination with other FEB species, causing head blight. *M. nivale* and *M. nivale* var. *majus* do not produce trichothecene mycotoxins. However, recent studies have shown that the presence of *M. nivale* in an FEB complex also including *F. culmorum* caused a significant effect on DON levels (Jennings *et al.*, 2000; Simpson *et al.*, 2001).



**Table 1.1** Common *Fusarium* species and their associated mycotoxins.

<i>Fusarium</i> Species	Mycotoxins
<i>F. avenaceum</i>	T-2 toxin, nivalenol (NIV), Deoxynivalenol (DON), Zeralenone (ZEN).
<i>F. culmorum</i>	DON, 3-acetyl DON, 15-acetyl DON (ADON), NIV, fusarenon X (FX), ZEN.
<i>F. graminearum</i>	DON, 15-ADON, NIV, FX, ZEN
<i>F. oxysporum</i>	Moniliformin, wortmannin, fusaric acid, sambutoxin
<i>F. poae</i>	T-2 toxin, HT-2 toxin, NIV, DAS, FX
<i>F. sambucinum</i>	Sambutoxin
<i>F. sporotrichioides</i>	T-2 toxin, HT-2 toxin, neosolaniol (NEO), diacetoxyscirpinol (DAS), FX, ZEN
<i>F. verticillioides</i>	Fumonisin, moniliformin, fusarin C
<i>F. proliferatum</i>	

### 1.3.3 Enumeration

Enumeration of *Fusarium* may be carried out on Potato Dextrose Agar (PDA) supplemented with chloramphenicol or another broad-spectrum antibiotic. On this medium *Fusarium* colonies will have the following appearance: Low to floccose colonies, coloured white, pink or purple. The underside of the colony will appear pink to red or purple. The *Fusarium* species used in this work form white colonies on PDA, which gradually turn red from the centre outwards as the colony ages. The shape of the macroconidia, microconidia, or presence/absence of chlamydospores may be used to identify most species of *Fusarium*.

### 1.3.4 *Fusarium culmorum*

*F. culmorum* spores and chlamydospores can survive in organic detritus of the soil for long periods of time. Warm, wet environments favour development of surface *F. culmorum* infection. The fungus can cause death of seedlings pre- and post-emergence. Stem lesions are often observed on seedlings which survive, and later brown lesions around the internode and node may occur at the base of the stem. The damage caused to the stem base is rarely severe. However, sometimes the stem rot will cause whitehead, an indication that the lowest nodes on the stem have become infected (Liggitt *et al.*, 1997).

*F. culmorum* is not normally associated with diseases of the leaves. However it will vigorously attack the ear causing the condition known as ear blight. Ear blight is most obvious when the ears are green, as infected florets or spikelets will appear bleached, possibly with pink biomass showing (Plate 1.1). If the rachis becomes infected all parts of the ear above this point appear bleached. If infection occurs soon after flowering no grain will form in the spikelets. In more developed ears the fungus will cause shrivelled grains and infect the grain directly.

It is believed that infection is spread up the diseased plant and transferred to its neighbours by rain-splashed or airborne sticky conidia. Birds have also been shown to





**Plate 1.1** FEB infection in a field of wheat (top left). The infected ears have turned a light brown. Ear of wheat showing signs of FEB infection (top right). Grains of wheat from an infected ear (bottom).



transport *Fusarium* spores, aiding dispersion of the fungus. Infection of wheat can be promoted by physical damage produced by insects such as the European corn borer *Ostrinia nubilalis* (Magg *et al.*, 2002). This is further aggravated by the action of insects such as the sap beetle, which is attracted to the wounds on the plant caused by other insects. Damage to grain in storage by insects is particularly problematic as it increases  $a_w$ . This creates “hot spots” within the dried stored grain where fungal infection may manifest.

*F. graminearum* and *F. poae* cause similar symptoms to those produced by *F. culmorum*. There are however, some differences. For instance, infection of the ear by *F. graminearum* produces vivid purple growth on the ear. This type of symptom earned it the name "scab". Also the mycotoxins produced by these *Fusarium* species are different.

*F. culmorum* and *F. graminearum* are both important wheat pathogens. These two species proliferate throughout the world. *F. culmorum* is dominant in cold regions with *F. graminearum* predominant in warmer areas. Therefore, FEB in the UK is most commonly caused by *F. culmorum*. However, a recent study (Jennings *et al.*, 2000) has found that *F. graminearum* is starting to gain a foothold in the UK, a region normally dominated by *F. culmorum*. Although *F. culmorum* and the other *Fusaria* are normally associated with pre-harvest diseases they are also capable of colonising and contaminating cereals post-harvest if conditions are conducive to fungal growth and mycotoxin production (Pixton, 1976; Magan & Lacey, 1988; Miller, 1995; Homdork *et al.*, 2000; Magan *et al.*, 2003).



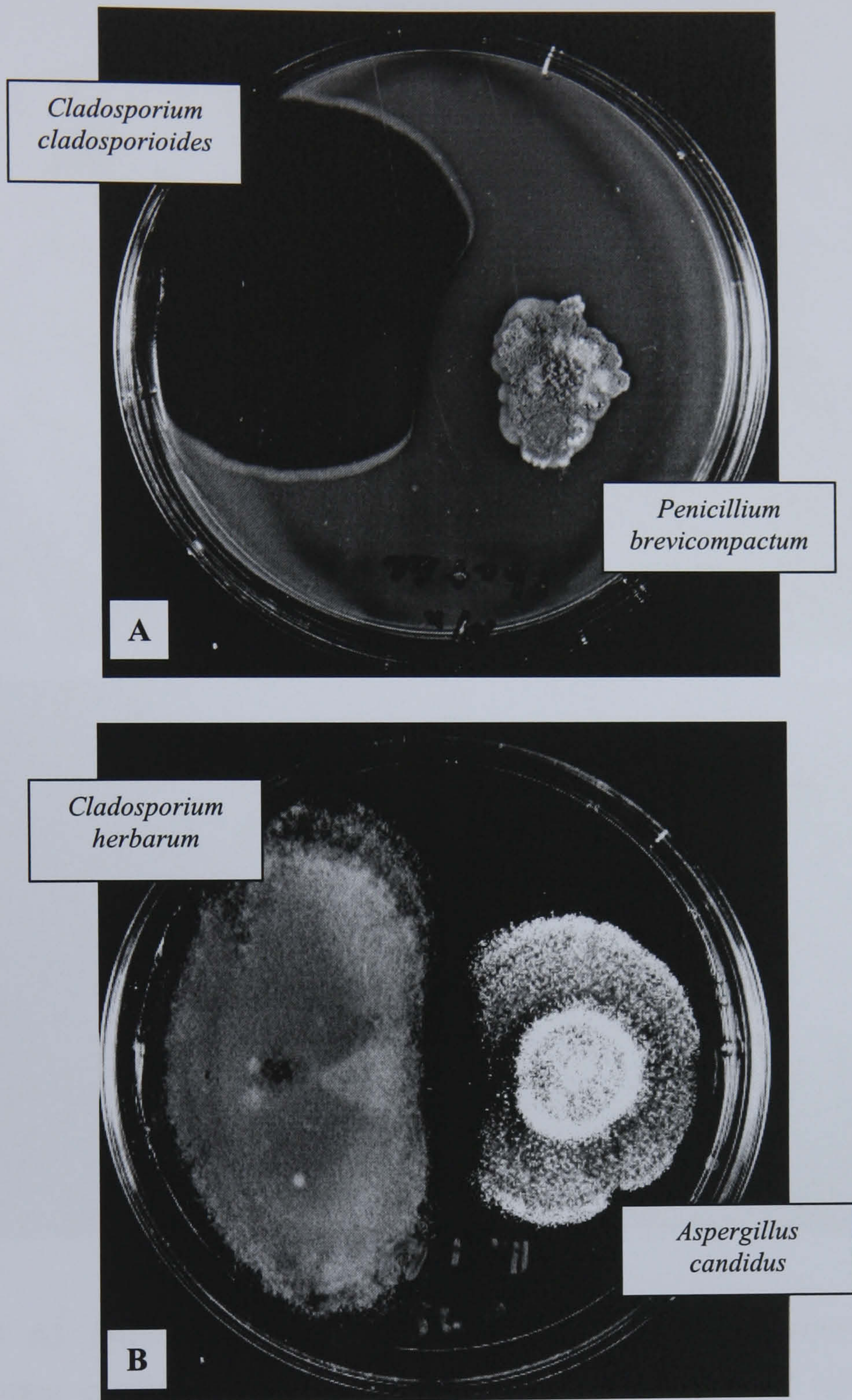
## 1.4 FUNGAL INTERACTIONS

Fungi are not normally found homogeneously in the field or on stored grain but in mixed populations. Fungal species growing in the same ecological niche in proximity to each other may exert influence over each other (Magan & Lacey, 1984; Rayner *et al.*, 1995; Marin *et al.*, 1997; Magan *et al.*, 1997; Marin *et al.*, 1998; Lee & Magan, 1999a; Lee & Magan, 1999b; Marin *et al.*, 2001). This influence may occur on hyphal contact or at a distance. Reactions over a distance are due to the action of exoenzymes and extracellular secondary metabolites produced by the fungi. Fungal interaction can produce changes to colony morphology at macro and microscopic level. Plate 1.2 and Plate 1.3 show some examples.

Changes in environment (i.e.  $a_w$ , temperature, fungicide) may cause a change in fungal interactions (Magan & Lacey, 1984; Marin *et al.*, 1998; Lee & Magan, 2000; Marin *et al.*, 2001). This causes problems if use of a fungicide leads to a toxigenic fungus gaining dominance. An example of this was recently observed in field trials (Jennings *et al.*, 2000; Simpson *et al.*, 2001). Evidence was found that suggested that the treatment of FEB caused by a mixed population of *M. nivale* and *F. culmorum* with azoxystrobin caused an increase in DON contamination. This was thought to be due to the inhibition of *M. nivale* by azoxystrobin allowing the resistant *F. culmorum* to colonise the ear unhindered.

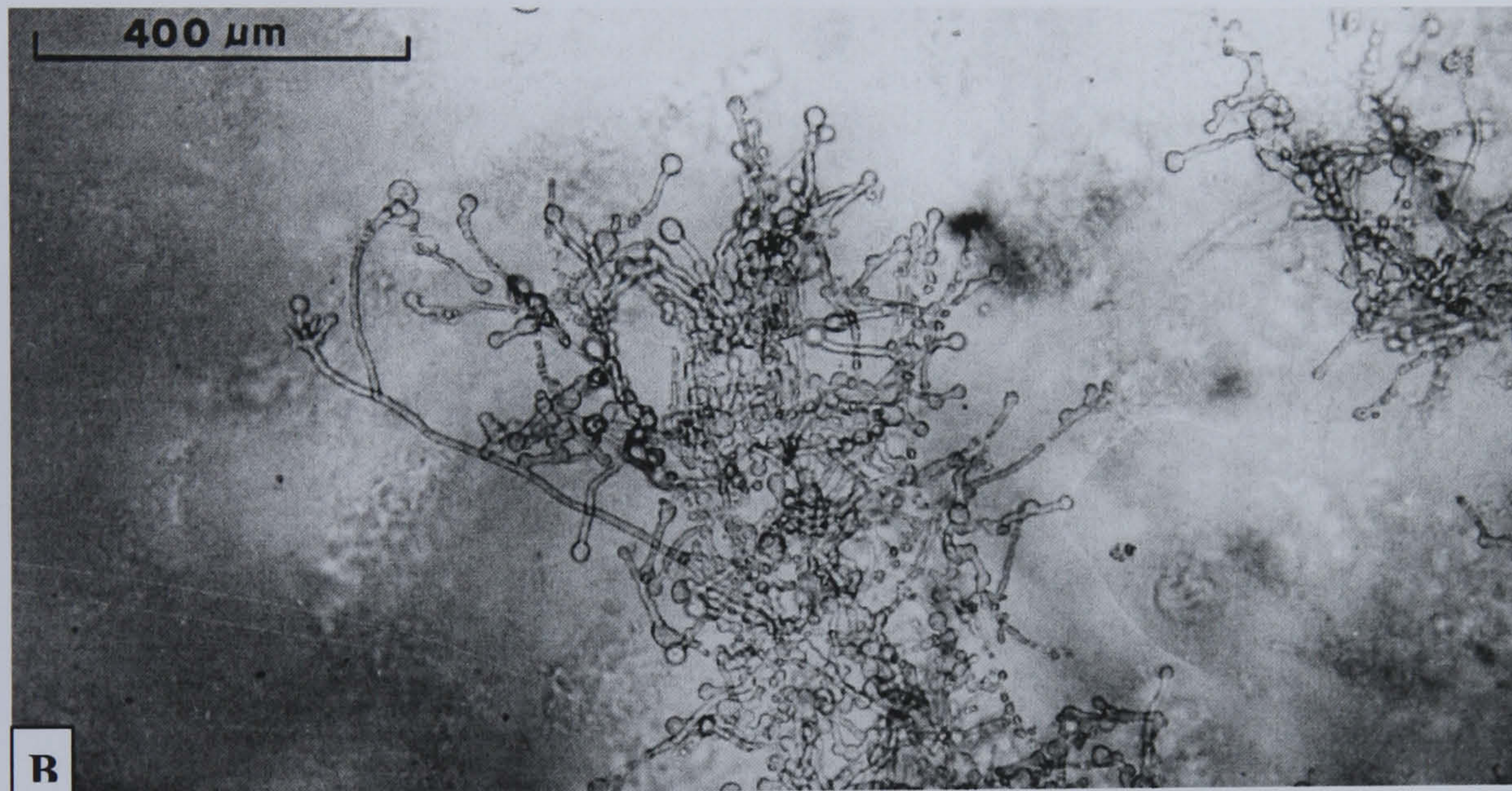
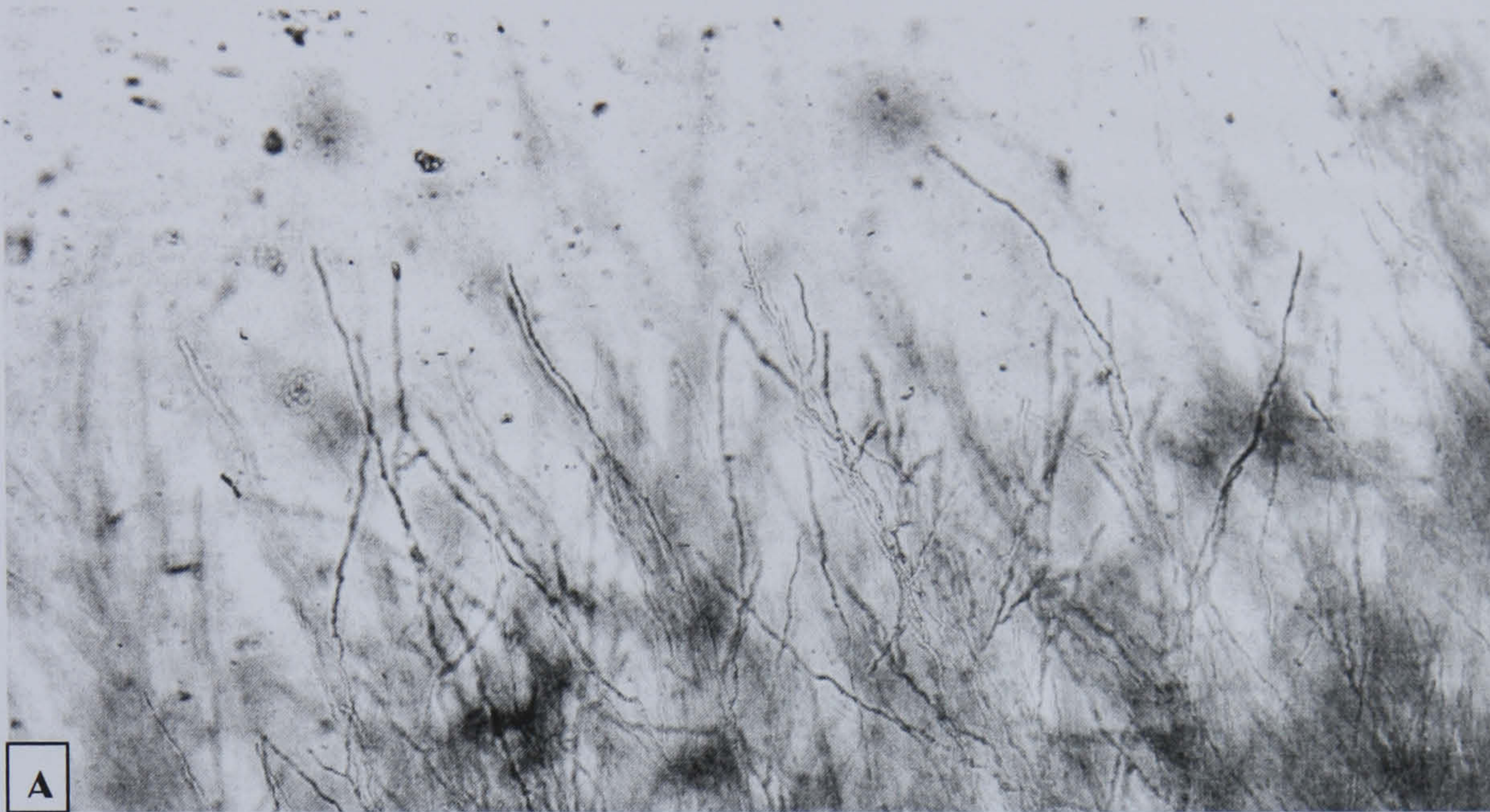
Fungal interactions may be measured qualitatively *in vitro*. Fungi are placed within a category determined by the type of interaction reactions - both macro and microscopically - they produce in the presence of another fungal colony. A numerical score represents each category. The more dominant the fungus is over its neighbour the higher the score. Interaction scores are affected by a number of variables, e.g. temperature,  $a_w$ , carbon sources, time, and substrate (Magan & Lacey, 1984). Interaction scores for one organism may be added together to form an Index of Dominance ( $I_D$ ). The  $I_D$  is used to compare the dominance of fungi under different environmental conditions from the same ecological niche. This has not been





**Plate 1.2** A). Dominance at a distance between *Penicillium brevicompactum*. and *Cladosporium cladosporioides*. B). Mutual inhibition on contact between *Aspergillus candidus* and *Cladosporium herbarum* from Magan and Lacey (1984).





**Plate 1.3** A). Dominance at a distance by *Penicillium brevicompactum*. over *Cladosporium cladosporioides*. B). Mutual inhibition between *A. candidus* and *Cladosporium herbarum* (Magan & Lacey, 1984).



previously used to try and understand the relative competitiveness of *Fusarium* species causing FEB.

Alternative approaches have also been utilised to understand the relative competitiveness of different species within fungal communities colonising grain. Willson & Lindow (1994; 1994), working with biocontrol systems, suggest that the co-existence of microorganisms particularly on plant surfaces may be mediated by nutritional resource partitioning. Thus *in vitro* carbon source utilisation patterns (Niche size) could be used to determine niche overlap indices (NOI) and thus the level of ecological similarity. Based on the ratio of the number of similar C sources utilised and those unique to an individual isolate or species, a value between 0-1 was obtained. NOI of  $> 0.9$  were indicative of coexistence between species in an ecological niche, while scores of  $< 0.9$  represented occupation of separate niches. This approach was modified by Marin *et al.*, (1998) and Lee & Magan (1999b) for a multifactorial approach by including water activity and temperature into the system. This demonstrated that based on the utilization of maize C sources, the NOIs for fumonisin-producing strains of *Fusarium verticillioides* and *Fusarium proliferatum* were  $> 0.9$  at  $> 0.96$  aw at 25 and 30 °C, indicative of co-existence with other fungi such as *Penicillium species*, *A. flavus* and *A. ochraceus*. However, for some species, pairing with *F. verticillioides* resulted in NOI values  $< 0.8$  indicating occupation of different niches. Nutrient status is very important. Lee & Magan (1999b) demonstrated that comparison of C sources in a standard BIOLOG test plate (95 carbon sources) with those only relevant to maize grain (18 carbon sources) gave very different results in terms of niche size and NOI under different environmental conditions. This approach confirms that interactions and dominance of component fungi in the community are dynamic, not static. This emphasises the importance of taking account of such fluxes in any integrated approach to understanding and controlling the activity of mycotoxigenic spoilage moulds in the stored grain ecosystem.



## 1.5 MYCOTOXINS

Mycotoxins are typical secondary metabolites, produced by filamentous fungi, and their biosynthesis involves the full range of secondary metabolic pathways, including the polyketide, mevalonate and amino acid derived pathways, as well as a combination of these (Moss, 1985). Mycotoxins cause a variety of mycotoxicoses in animals (including humans). *Fusarium* species produce a wide array of mycotoxins. The type and concentration of mycotoxins produced varies with species and environmental conditions. These mycotoxins contaminate the grain and straw of wheat. The effect on health of mycotoxin poisoning varies from acute to chronic dependent on type and dose. Table 1.2 lists the conditions caused by some common mycotoxins.

### 1.5.1 *Fusarium* mycotoxins

There are three classes of *Fusarium* mycotoxins that are of particular importance to human and animal health:-

- Trichothecenes (DON, NIV, T2, HT2)
- Zearalenone (ZEN) and its derivatives.
- Fumonisins

*F. graminearum* and *F. culmorum* produce deoxynivalenol (DON), Nivanenol (NIV) or Zearalenone as a primary toxic metabolite. Which of the three toxins are produced varies with isolate. It is often found that isolates from the same geographic region will produce the same mycotoxin. However, this project is primarily concerned with DON production as this is the most common mycotoxin produced by *F. culmorum*.

**Table 1.2** Major mycotoxin and toxin producing fungi from corn, cereals, soybeans, peanuts, and other products and some of their effects on animals (Jacobsen *et al* 1993).

Genus	Toxin	Example of Fungal source	Feeds or Food Effect	Possible Effects on animals
<i>Aspergillus</i> species	(primary) Aflatoxins B1, B2, G1 and G2 B2a, G2a, M1 and M2 are metabolites and seldom present in grain; M1 and M2 are important contaminants in milk	<i>A. flavus</i> and <i>A. parasiticus</i>	Cereal grains, peanuts, soybean and other foods	Hepatotoxin; carcinogenic; reduced growth rate; haemorrhagic enteritis; suppression of natural immunity to infection; decreased production of meat, milk and eggs.
	Ochratoxins (nephrotoxins)	<i>A. ochraceus</i> and <i>Penicillium viridicatum</i>	Cereal grain	Toxic to kidneys and liver; poor feed conversion, reduced growth rate. General unthriftiness; reduced immunity to infection
	Sterigmatocystin	<i>A. ridulans</i> and <i>A. versicolor</i>	Cereal grains	Toxaemia; carcinogenic
	Tremorgenic toxin	<i>A. flavus</i> , <i>P. cyclopium</i> and <i>P. palitans</i>	Cereal grains, soybeans, peanuts and other food feeds	Tremors and convulsions
<i>Penicillium</i> spp.	(primary) Luteoskyrin	<i>P. islandicum</i>	Rice	Tremors and convulsions
	Patulin	<i>P. urticae</i> , <i>P. expansum</i> , <i>P. claviforme</i> , and <i>A. clavatus</i>	Cereal grains, apple products	Haemorrhages of lung and brain; edema toxic to kidneys; possible carcinogenic
	Rubratoxin	<i>P. rubrum</i>		Liver damage and haemorrhage
	Citrinin	<i>P. citrinum</i>		Kidney damage



**Table 1.2** (continued). Major mycotoxins and toxin producing fungi from corn, cereals, soybeans, peanuts, and other products and some of their effects on animals (Jacobsen *et al.*, 1993).

Genus	Toxin	Example of Fungal source	Feeds or Food Effect	Possible Effects on animals
<i>Fusarium</i> species	Zearalenone (estrogenic syndrome)	<i>F. graminearum</i> , <i>F. tricinctum</i> , and <i>F. moniliforme</i>	Cereal grains	Hyperoestrogenism , infertility, stunting and death
	Zearalenol			
	Emetic or feed refusal (vomitoxin), deoxynivalenol, similar symptoms caused by nivalenol	<i>F. graminearum</i> , <i>F. culmorum</i>	Cereal grains	Food refusal, reduction in weight, reproductive dysfunctions, immune suppression
	Other trichothecenes (T2 HT2 monoacetoxyscripenol or MAS, Diacetoxyscripenol or DAS	<i>F. graminearum</i> , <i>F. tricinctum</i> , <i>F. poae</i> , and <i>F. sporotrichioides</i>	Cereal grains	Inflammation of GI tract and possible haemorrhage; edema; vomiting and diarrhoea; infertility; degradation of bone marrow; weight reduction; slow growth; sterility
	Fumonisin B1, B2	<i>F. moniliforme</i>	Corn	Leucoencephalomalacia, “blind staggers” in horses.
Ergot	Ergopeptines	<i>Claviceps purpurea</i>	Cereal grains	Vasoconstriction, loss of extremities (i.e ears, arms etc)
	Ergovaline	<i>Acremonium coenophialwn</i>	Fescue	Reduced weight gain, abortion, poor survivability of offspring, fescue foot

### 1.5.2 Deoxynivalenol

DON is widely distributed in food and feed. It occurs virtually wherever cereals are grown with the exception of dry land wheat production (i.e. Australia) (IARC, 1993). DON is produced by either a 3 or 15 acetylated precursor, known as acetyl deoxynivalenol (ADON). 15 acetylated precursor is normally found from isolates from South/North America and the 3 acetylated precursor from Europe and Asia. NIV is also produced by some *Fusarium* species. It has a similar chemical composition as DON and is able to cause similar mycotoxicoses. Figure 1.2 shows the chemical structure of DON and NIV.

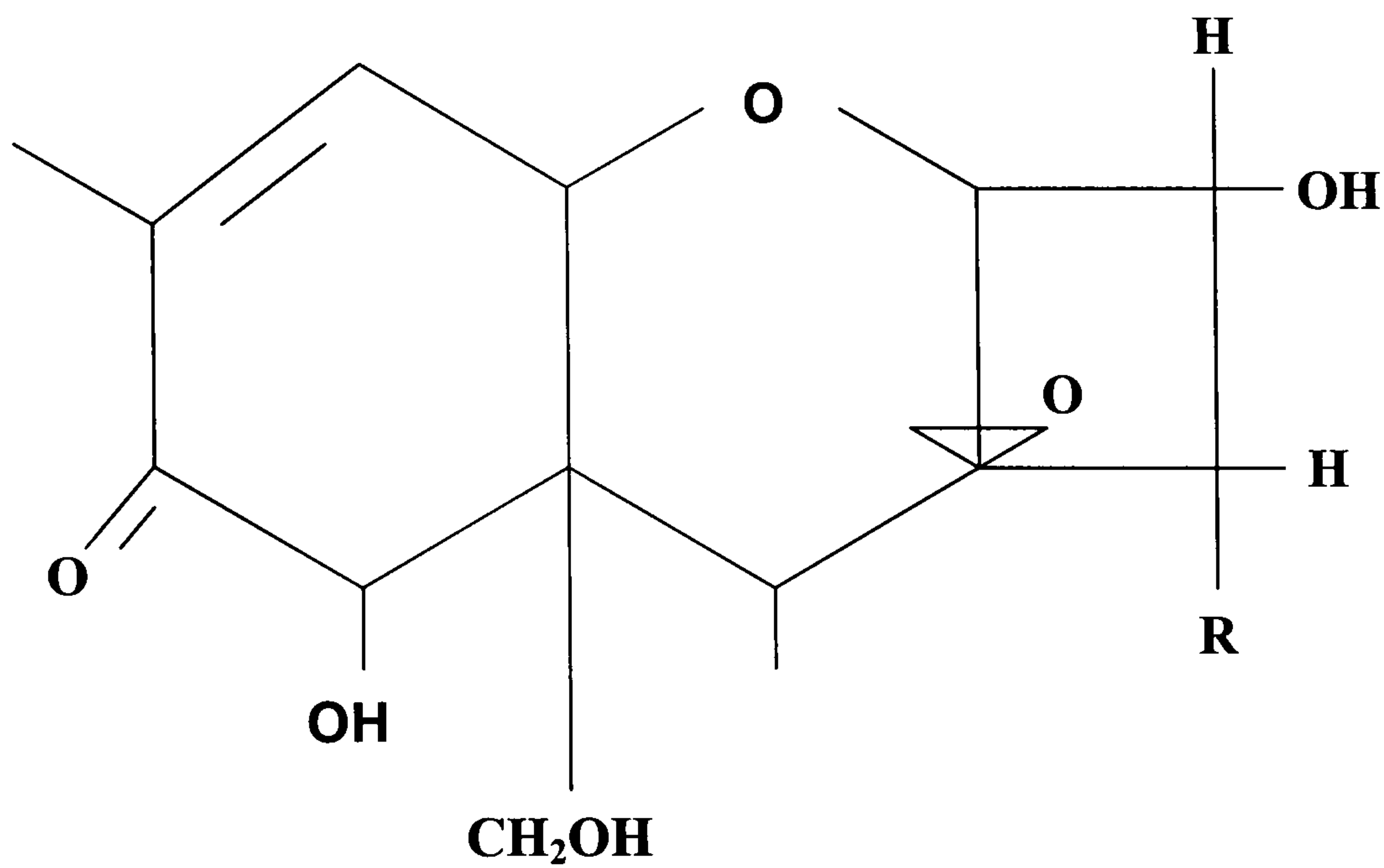
Swine are the domesticated animal most sensitive to DON mycotoxin. Acute toxicity manifests as intestinal disorder and emesis, which is where the common name for DON, 'Vomitoxin' originates. Acute DON conditions are rare though, as swine will refuse to eat contaminated feed. Feed refusal starts to occur at  $1 \mu\text{g g}^{-1}$  of DON. This results in a reduction or halt in weight gain by the livestock. At low levels DON also causes a variety of reproductive dysfunctions in swine including:-

- Abortion
- Stillbirths
- Weak offspring

Poultry have a greater tolerance to DON than swine. However, low levels of DON do have a detrimental effect on egg quality and weight. Cows also show a greater tolerance to DON than swine. This is possibly due to extensive degradation of secondary metabolites, which occurs in the rumen. The effect of DON on cattle includes.

- Feed reduction.
- Reduced conception rate.
- Possible effect on milk production.





**DON: R = H**

**NIV: R = OH**

**Figure 1.2.** Molecular structure of deoxynivalenol (DON) and nivalenol (NIV).



DON has also caused large scale human toxicosis. For instance, a large number of people were poisoned by DON in the Kashmir Valley of India in 1987, after consuming bread made with mouldy flour. There have also been other cases in India, Japan and Korea related to the consumption of mouldy maize and wheat. (Jacobsen *et al.*, 1993). In Europe, DON levels are unacceptably high in Germany and Sweden (Schollenberger *et al.*, 1999; Birzele *et al.*, 2000; Ellner, 2000; Schollenberger *et al.*, 2002).

DON and the trichothecenes in general have been shown to elicit a number of detrimental immunological effects in laboratory animals. This resulted in an increase in the animals' susceptibility to bacterial, viral and fungal disease. Unfortunately little is known about the long term impact of exposure to DON on the immune system of humans.

### **1.5.3 Interactions between mycotoxins**

As mentioned earlier, fungi do not normally synthesise only one type of mycotoxin or secondary metabolite but a wide range. Therefore cereals contaminated by *Fusarium* species may have a cocktail of mycotoxins present. In nature this situation is further complicated by the action of other fungal species, their associated secondary metabolites and the resulting interspecific and intraspecific interactions. The interactions of these mycotoxins/secondary metabolites on health are not as well documented as the effects of the individual agents (Tajima *et al.*, 2002).

Mycotoxin interactions have been shown to make a mycotoxin dangerous to an animal that was previously immune to its effects, and in others, lower doses of mycotoxin were able to elicit a response when supplemented with other secondary metabolites. For instance, fusaric acid enhances *Fusarium* mycotoxin action. Fusaric acid with FB1 was shown to be toxic to fertile chicken eggs. Individually the agents had no effect. Table 1.3 (D'Mello *et al.*, 1999) shows some of the *Fusarium* mycotoxin interactions which have been determined. Synergistic effects have been proven to exist for DON + fusaric acid and DON + FB1.



**Table 1.3** Various mycotoxicoses caused by the combined effect of two mycotoxins or secondary metabolites in domestic animals (D'Mello *et al.*, 1999).

Source of mycotoxins	Combination	Animal Species	Responses	Interaction
Naturally contaminated	DON and fursaric	Pigs	Growth	Synergistic
Inoculated maize and pure mycotoxin	DON and T-2 Toxin	Pigs	Feed intake and growth	Adverse effects of DON reduced at ..
Contaminated wheat and	DON and FB1	Pigs	Weight gain	Synergistic
Pure mycotoxin	T-2 tooxin and OA	Pigs	Feed intake and weight gain	Additive
Culture material and pure mycotoxin	DON and MON	Broiler chicks	Feed intake and weight gain	Less than additive
Culture material and contaminated wheat	DON and fumonisins	Broiler chicks	Weight gain	Less than additive
Culture material and pure	T-2 toxin and	Broiler chicks	Weight gain	Additive
Pure mycotoxins	FB1 and	Broiler chicks	Mortality	Additive
Pure mycotoxin and culture material	T-2 toxin and fumonisins	Turkey poults	Weight gain, certain blood and enzyme	Additive
Culture material and pure	DAS, fumonisins or	Turkey poults	Weight gain	Synergistic
Inoculated rice and pure mycotoxin	DAS and aflatoxins	Lambs	Weight gain and serum $\gamma$ -glutamyl	Synergistic or additive, less than



#### 1.5.4 Residues

Mycotoxins may carry over into animal produce such as milk and eggs. This poses a possible threat to health, and another route by which mycotoxins may enter the food chain. It has been demonstrated that hens fed grain contaminated with radio-labelled DON produced eggs with low levels of the mycotoxin present. Residues of the mycotoxin were seen to decline when the contaminated feed was removed.

#### 1.5.5 Regulations

*Fusarium* mycotoxins are not currently regulated. However, tolerance limits are available in literature (Dutton, 1996; D'Mello *et al.*, 1999; Ellner, 2000; Sudakin, 2003). D'Mello *et al.* (1999) discusses some of the problems in determining mycotoxin tolerance levels from scientific research. Dose in terms of intake is more important than dietary levels. Rate of detoxification of absorbed toxins is also an important factor in establishing recommended tolerance levels. There were a number of complications when compiling this data. Variation in methodology and test criteria (i.e. what dictates a positive result) produces conflicting results. No allowance was made for mycotoxin interactions, thus resulting in discrepancies between experiments where samples had been contaminated with *Fusarium*, and those which used samples contaminated with the pure mycotoxin. EU proposed limits – raw cereals on  $1500 \mu\text{g Kg}^{-1}$  and  $1000 \mu\text{g Kg}^{-1}$  processed material for human consumption.

### 1.6 CURRENT CONTROL METHODS FOR *FUSARIUM* COLONISATION AND ASSOCIATED MYCOTOXINS

Wheat production was increased in the UK during the 1980s. An increase in FEB infection occurred in parallel with this (Bateman & Murray, 2001). A similar pattern of increase of FEB infection with increasing wheat production was also observed in



Canada (Sutton, 1982). The increased occurrence of FEB generated a large interest in methods of controlling *Fusarium*.

Although this study is concerned with the effect of fungicides, it should be mentioned that there are additional and alternative measures that may be taken pre- and post-harvest to reduce the risk of mycotoxin formation. Pre-harvest measures are often the easiest and cheapest to implement. Possible preventative measures that may be employed are; selection of disease resistant or less susceptible crop varieties, crop rotation, sufficient tillage management and fertilisation on demand (Ellner, 2000). The difference in cultivar susceptibility and DON production has been shown to be significant. For instance in one study 7.7 mg kg<sup>-1</sup> DON, was found on less susceptible cultivars, compared to 50.2 mg kg<sup>-1</sup> for susceptible strains (Ellner, 2000). Use of tight crop rotation schemes has been shown to yield higher mycotoxin concentrations (Teich & Nelson, 1984). Use of conservation tillage may result in greater levels of inoculum remaining in the topsoil than with more aggressive tillage regimes (Ellner, 2000; Kladvko, 2001). The amount of inoculum present is important as it directly relates to disease severity (Zadoks & Schein, 1979).

### **1.6.1 Fungicides as *Fusarium* control agents**

A large number of fungicides have been used to control *Fusarium*. Non-systemic fungicide mixtures based on prochloraz were used but have in the majority of cases been replaced by systemic fungicides, for instance Tridemorph, Carbendazin and Zineb were used to control FEB in the 1980s (Moss, 1985; Moss & Frank, 1985; Magan & Lacey, 1986). Although these fungicides controlled FEB, some had unforeseen effects on mycotoxin production as can clearly be seen by some of the information found in literature; Tridemorph was shown to increase T2 production by *F. sporotrichioides* in a manner which was very dependent on concentration (Moss & Frank, 1985). At relatively low concentrations of 6-8 ppm tridemorph causes a small enhancement of growth while considerably reducing T-2 toxin and diacetoxyscirpenol production. By contrast, concentrations which inhibited growth by about 50% (30-50 ppm) stimulated formation of the T2 toxin (Moss & Frank, 1985). Fernando and Bean (1986) found a similar trend for *Aspergillus flavus*. Tricyclazole and benzothiazole



derivatives inhibit alfatoxin production at concentrations that had produced only a small reduction in mycelial growth (Fernando & Bean, 1986). Maneb used on maize controlled both *F. graminearum* growth and ZEN production (Daughton & Churchville, 1985). Hasan (1993) compared the efficiency of dicloran, iprodione and vinclozolin at controlling *F. graminearum* and its toxins. Vinclozolin prevented DAS production at 500  $\mu\text{g ml}^{-1}$ , and at 250  $\mu\text{g ml}^{-1}$  ZEN synthesis was inhibited. However ZEN production was not inhibited by vinclozolin at a concentration of 500  $\mu\text{g ml}^{-1}$ . Matthies *et al.*, (1999) screened fungicides commonly used in Germany to control FEB (benomyl, thiabendazole, prochloraz, tebuconazole, tridemorph and fenopropimorph). The latter two fungicides did not elicit an effect on the production of 3-ADON *in vivo* or *in situ*. All the other fungicides at 0.5-1.0  $\mu\text{g ml}^{-1}$  inhibited 3-ADON production. At sub-optimal levels (0.1  $\mu\text{g ml}^{-1}$ ) of tebuconazole, mycotoxin production was stimulated by up to four times that of the control. In another study in Germany (Ellner, 1997) azole based fungicides, tebuconazole and metaconazole were found to be unable to control FEB and DON production by > 50%. Temperature was shown to be an important factor by D'Mello *et al.* (1998) on the stimulation of mycotoxin by difenoconazole. At 25 °C using sub-optimal levels of difenoconazole against *F. culmorum* mycotoxin production was increased and growth was unaffected. However, at 11 °C there was no mycotoxin stimulation. It was also found that the production of ADON was higher in controls using strains resistant to the fungicide.

As is evident from the examples above, the effects of fungicides on mycotoxin production is complex and varied. A fungicide that controls the growth of one species may have an undesired effect on mycotoxin production. Of more concern, however, is the stimulation of mycotoxin production when there is little or no control of growth. This coupled with *Fusarium* species (i.e. *F. culmorum*) which are resistant to many fungicides currently available has stimulated interest in new fungicidal agents and methods of control (Hope *et al.*, 2000; Magan *et al.*, 2002).



### 1.6.2 Essential oils as fungicidal or fungistatic agents

Herbs and spices have been used to treat illness or preserve food for centuries. Recent studies have attempted to determine the efficiency of extracts from selected plants as antimicrobial and antifungal agents.

Evidence has shown that some essential oils can control growth rate and spore germination of spoilage fungi. For example, Paster *et al* (1990) found that oregano and thyme essential oils inhibited the growth of *Aspergillus niger*, *A. ochraceus* and *A. flavus*. Growth was fully inhibited at 400  $\mu\text{g ml}^{-1}$  for oregano, and at 600  $\mu\text{g ml}^{-1}$  for thyme. At these concentrations the oils also prevented spore germination. Oregano and thyme also inhibited the growth of *Staphylococcus aureus* and *Salmonella typhimurium*. The oils of bay, cinnamon, clove and thyme were found to be effective inhibitors of five important food spoilage bacteria by Smith-Palmer *et al.* (1998). The oils were found to have a bacteriostatic concentration of 0.075 % or less. Yin (1998) investigated the antifungal properties of water-soluble extracts from traditional Chinese ingredients on the growth of *A. niger* and *A. flavus*. Garlic bulb, green garlic, and green onions were shown to have an inhibitory effect against these two fungi. Cinnamic acid is a phenolic component of several spices, including cinnamon. Cinnamic acid is used as a component in several food flavourings and by the Japanese as an antimicrobial agent in fish paste (Shimada *et al.*, 1991). Studies have found that cinnamic acid was particularly effective at controlling yeasts and moulds at acidic pH (Roller, 1995).

The modes of action of essential oils are largely unknown. However, there is discussion in the literature towards this end. In a study of Egyptian essential oils by Farag *et al* (1989) it was concluded that there was a relationship between the chemical structure of the main component of an essential oil and its antimicrobial activity. The inhibitory effect of the oils was generally attributed to the presence of an aromatic nucleus containing a polar functional group. This is a similar structure to that of phenols and chlorophenols, which are widely used in disinfectants. The presence of phenolic OH groups able to form hydrogen bonds with the active sites of target



enzymes was thought to increase antimicrobial activity. It was also stressed that the inductive effect of isopropyl groups should also be taken into consideration.

Studies have been conducted to determine the antimicrobial effect of essential oil active components. There have been conflicting results. Limonene and terpinene are the two main components of carrot seed oil. The inhibition of *Aspergillus parasiticus* was found to be higher for the components than for the complete oil (Batt *et al.*, 1983). In contrast the oils derived from orange or lemon peel were more effective at controlling growth and aflatoxin production than d-limonene, the main constituent of the two peel oils (Alderman & Marth, 1976). Cinnamaldehyde and eugenol, the major constituents of cinnamon and clove oils were found to inhibit mould growth (Bullerman *et al.*, 1977). Paster *et al.* (1995) found that thyme essential oil was more inhibitory than the individual components. It was suggested that the essential oil activity resulted mainly from synergistic or cumulative effects existing between the components. However, more information on the mode of action employed by oils is required to identify such possible synergism.

### **1.6.3 Antioxidants as fungicidal or fungistatic agents**

Antioxidants have been demonstrated to inhibit fungal growth (Thompson, 1991; Thompson, 1994; Torres *et al.*, 2003); Thompson (1993) studied the effects of esters of p-hydroxybenzoic acid (Paraben) on the growth of three mycotoxigenic fungi. Butyl and propyl paraben were the most effective, causing complete mycelial inhibition at concentration of 1.0-2.0 mM. Synergistic effects between parabens in combination had a pronounced effect on *F. graminearum*. It is known that the effectiveness of the parabens increases with an increase in the chain length of the ester group. However the mechanism of action of the parabens which produces inhibition of mycelium growth of toxigenic fungi is not clear. However in these studies the impact of environmental factors on efficacy of antioxidants on growth or mycotoxin production was not investigated.

The phenolic antioxidant butylated hydroxyanisole (BHA) prevented the germination of conidia of *Aspergillus* species on PDA at concentrations of 200  $\mu\text{g ml}^{-1}$  and above



(Thompson, 1992). Thompson (1992) concluded that BHA and other phenolic antioxidants and propyl paraben were the most effective inhibitors of mycelial growth.

The mechanisms behind this activity may be quite varied. Organic acids such as propionic, benzoic and sorbic acids have long been used to control fungal spoilage of food and the mode of action has been attributed to two factors; depression of intracellular pH by ionisation of the undissociated acid molecule or distribution of substrate transport by alteration of cell membrane permeability. Benzoic acid has also been shown to inhibit the cell wall, degrading enzymic activity in bacteria. Propyl paraben and BHA appear to work mainly at the cell membrane eliminating the pH component of the protomotive force and affecting energy transduction and substrate transport. BHA has also been shown to have a direct effect on the mitochondrial electron chain of trypanosomes, thus inhibiting respiration (Khan *et al.*, 2001).

## **1.7 HAZARD ANALYSIS CRITICAL CONTROL POINT (HACCP)**

The information gathered by this project will be used to help construct a HACCP scheme which will improve current procedures and means for controlling *Fusarium* species and associated mycotoxins.

Hazard analysis critical control point (HACCP) is a system for identifying, evaluating and controlling hazards, which are significant for food safety. The critical control points (CCP) within a HACCP scheme are points within the production process where food safety hazards may be prevented or controlled.

There are several objectives that a HACCP scheme must accomplish if it is to be effective:-

- Conduct a Hazard Analysis.
- Determine CCPs.
- Establish CCP critical limits.



- Establish a system to monitor control of the CCPs.
- Establish corrective actions if CCP is not under control.
- Establish procedures to verify that HACCP is working effectively.
- Establish documentation concerning all relevant procedures and relevant procedure and records.

The hazard analysis considers possible detrimental events and the risk of them occurring. Once hazards have been determined, the points within the production process where these hazards can be controlled or prevented are identified. At this point it is useful to visualise the manufacturing process by use of flow diagrams. The CCPs can also be added to the flow diagrams. Figure 1.3 and 1.4 shows the rough outline for the production process of grain to the consumer. Figure 1.5 shows the critical control points which are present in the system.

The critical limit applied to each CCP is a point at which the hazard is likely to manifest, thus reducing the product quality. For instance if the HACCP scheme is designed to prevent mycotoxin contamination of stored wheat grain, a CCP would be the moisture content of the stored grain. The critical limit of the CCP would be a moisture content at which it has been proven that mycotoxin levels do not exceed the levels set out by law or work practices. The critical limits may vary depending on the end use of the product. For instance, grain intended for human consumption will have lower critical limits than grain for animal feed as the mycotoxin level allowed in food for human consumption is generally less than for animal feed. Information required to determine critical limits for DON/NIV production by *F. culmorum* or *F. graminearum* is not currently available.



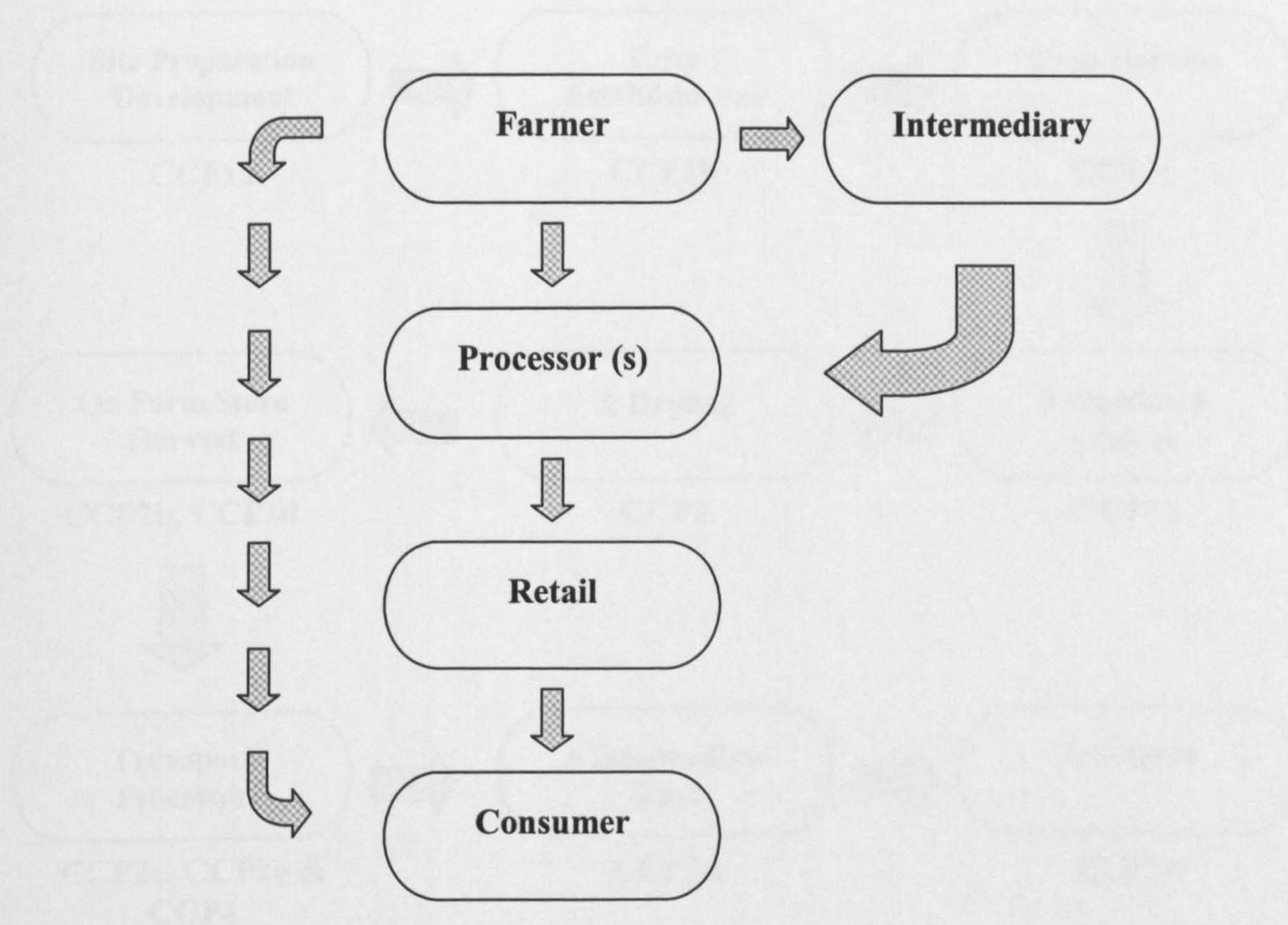


Figure 1.3. Grain supply chain.

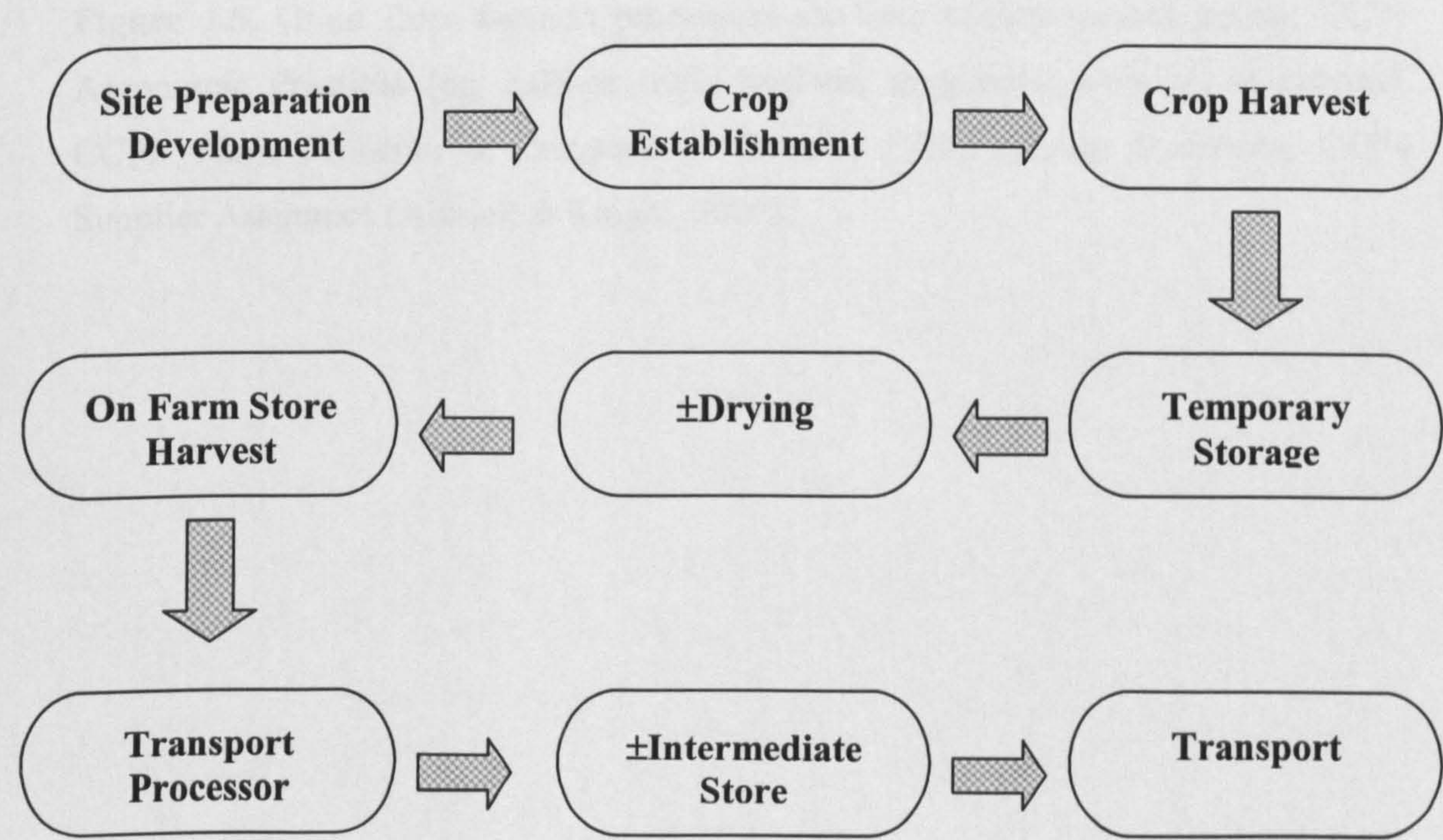
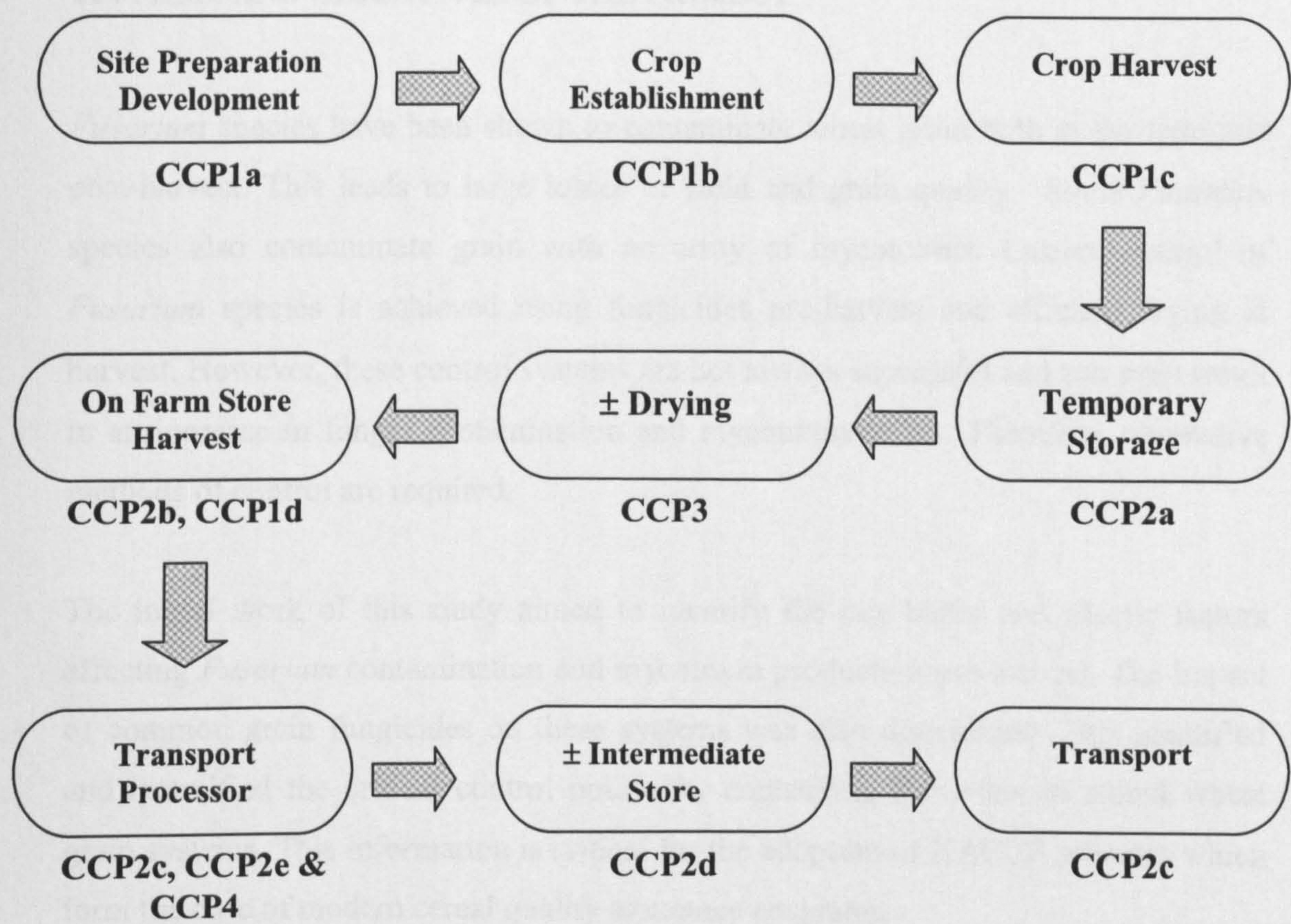


Figure 1.4 Grain from farm to processors.





**Figure 1.5.** Grain from farm to processors showing critical control points; CCP1 Agronomic Practices (eg. cultivar, field hygiene, fungicides, moisture at harvest), CCP2 Time, Moisture & Temperature Control, CCP3 Drying Dynamics, CCP4 Supplier Assurance (Aldrick & Knight, 2000).



## 1.8 AIMS AND OBJECTIVES OF THE PROJECT

*Fusarium* species have been shown to contaminate wheat grain both in the field and post-harvest. This leads to large losses in yield and grain quality. Some *Fusarium* species also contaminate grain with an array of mycotoxins. Current control of *Fusarium* species is achieved using fungicides pre-harvest and efficient drying at harvest. However, these control systems are not always successful and can even result in an increase in fungal contamination and mycotoxin levels. Therefore, alternative methods of control are required.

The initial work of this study aimed to identify the key biotic and abiotic factors affecting *Fusarium* contamination and mycotoxin production pre-harvest. The impact of common grain fungicides on these systems was also determined. This identified and quantified the critical control points for controlling *Fusarium* in stored wheat grain systems. This information is critical for the adoption of HACCP schemes which form the core of modern cereal quality assurance programs.

The second part of the study focused on the control of *Fusarium* species post-harvest. Natural antifungal agents in the form of food grade essential oils and antioxidants were screened and tested rigorously to identify new control agents for *Fusarium* colonisation and mycotoxin production.

This project focused primarily on the *Fusarium* contamination present in UK wheat grain. Therefore, *F. culmorum* and *F. graminearum* and their most common mycotoxins DON and NIV were studied in most depth as these fungi are the most frequently isolated *Fusarium* species on UK wheat grain.

Aspects of this work provided important information for a European Union wide project “Prevention of *Fusarium* mycotoxins entering the food chain” (QLK1-CT-1999-00996).

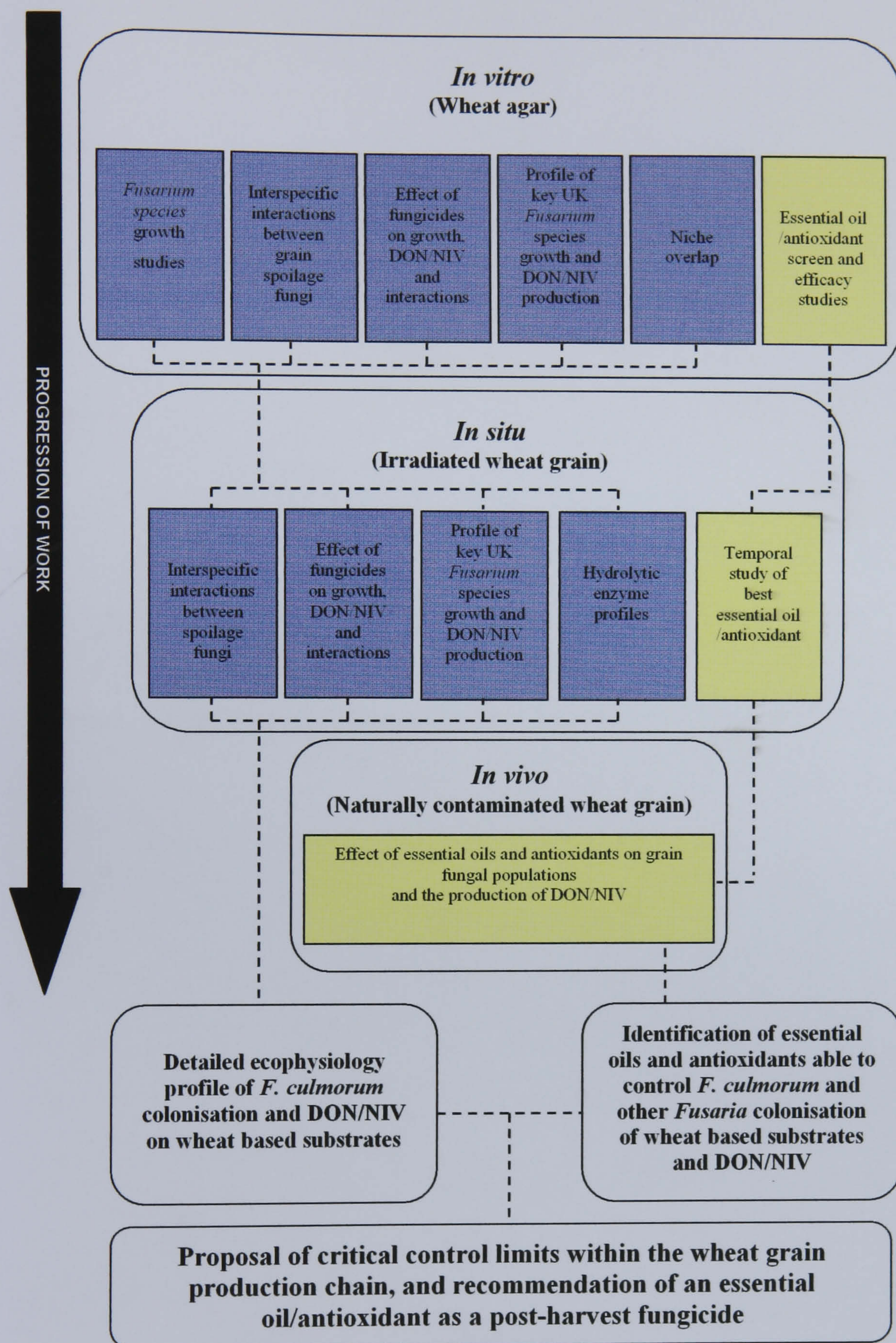


The key objectives of this study were: -

1. Compare growth of *F. culmorum* isolates from the UK and northern Europe under different environmental conditions.
2. Construct temporal two-dimensional profiles for colonisation and DON/NIV production by *F. culmorum* and *F. graminearum* on wheat based substrates.
3. Investigate the effects of interspecific interactions on colonisation and DON/NIV production by *F. culmorum*.
4. Determine Niche Overlap dominance and hydrolytic enzyme production profiles for *F. culmorum* and other wheat grain spoilage fungi.
5. Study the effects of common wheat fungicides on the colonisation of *F. culmorum* and the effect of sub-optimal levels of these fungicides on interspecific interactions and DON/NIV levels.
6. Screen food grade essential oils and antioxidants for the control of *F. culmorum*.
7. Study essential oils and antioxidants identified in the screen in more depth *in vitro* on wheat agar and *in situ* on irradiated wheat grain. Determine their efficacy for control of *F. culmorum*, *F. graminearum*, and *F. poae* colonisation and mycotoxin (DON, NIV, T2 and HT2) production.
8. Investigate the ability of the essential oils and antioxidants to control fungal populations and DON/NIV levels in naturally contaminated wheat grain.

Figure 1.6 summaries the components of this work, their order and interactions.





**Figure 1.6** Flow diagram of the experimental work carried out in this thesis. Dotted line represents experimental interconnectivity.



# CHAPTER 2

## MATERIALS AND METHODS



## 2.1 ORGANISMS

All of the fungi used in this work were isolated from wheat grain. The majority of strains were isolated from wheat grain originating in the UK; however, some of the *F. culmorum* isolates were from other European countries. Table 2.1 lists all of the isolates used throughout the study.

Fungal isolates were kept in -80 °C storage as spore or mycelial suspensions in a 15 % glycerol solution as a cryo-protectant. Isolates were also kept as slopes or plates on malt extract agar (MEA) and synthetic nutrient agar (SNA) for up to 3 months. Purity of storage cultures was verified by streak plating and microscopic analysis. The *Fusarium* isolates used were previously confirmed to produce mycotoxins.

Isolates were retrieved from frozen storage by thawing an ampoule of spore suspension and then spread plating 100 µl of the suspension on to MEA and SNA plates. These plates were then incubated at 15 or 25 °C. If conidial production was required the *Fusarium* cultures were exposed to backlight for 8 hours per day of incubation.

## 2.2 MEDIA AND SUBSTRATES

The majority of the in vitro experiments utilised a wheat-based agar medium, referred to as wheat agar hereafter. This was prepared by milling wheat grain into a fine powder and then adding the appropriate amount (20 g litre<sup>-1</sup> was used throughout the study) to a 2 % agar (Oxoid technical agar no:1) solution. This was then shaken and autoclaved at 120 °C for 15 mins. The sterilized medium was allowed to cool to approx 40 °C before pouring into 90 mm Petri dishes. The medium had to be agitated during pouring to ensure an even distribution of the insoluble materials throughout the medium. MEA (Oxoid) was used for the cultivation of stock cultures and was made in accordance with the manufacturer's instructions. Stock cultures of *Fusarium* and *Microdochium* species were also kept on SNA. One litre of SNA requires the



**Table 2.1** Strains and isolate information for fungi used during this study.

Species	Isolates (origin or ID number/authorities)
<i>Alternaria tenuissima</i>	IBT 8320 (T.U. Denmark)
<i>Cladosporium herbarum</i>	IBT 7961 (T.U. Denmark)
<i>Fusarium culmorum</i>	UK1(CC171), UK2(CC68), UK3(98WW4.5FC, Rothamsted Research Culture Collection, Harpenden, Herts, U.K.) <sup>1,2</sup> , ITA1(F.C418, ISPAVE) , ITA2(F.C137, ISPAVE) ITA3(F.C1169, ISPAVE), NOR1, NOR2, NOR3
<i>Fusarium graminearum</i>	Isolated from UK wheat grain <sup>1,2</sup>
<i>Fusarium poae</i> *	Isolated from UK wheat grain
<i>Microdochium nivale</i>	18/1/N
<i>Microdochium nivale</i> var. <i>majus</i>	1/1M
<i>Penicillium verrucosum</i>	M450 (Dierckx, Sweden) <sup>3</sup>

1. DON producer.

2. NIV producer.

3. OTA producer.

\* *powdery poae* (equivalent *Fusarium langsethiae*, refered to through out this work as *Fusarium poae*)



following 1.0 g  $\text{KH}_2\text{PO}_4$ , 1.0 g  $\text{KNO}_3$ , 0.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g  $\text{KCl}$ , 0.2 g Glucose, 0.2 g Saccharose, 20 g agar, 1000 ml distilled water.

Naturally contaminated or irradiated wheat grain was used in this study. Wheat grain was irradiated with 12KGrays of gamma radiation. This killed all microflora present in the grain, however the germinative capacity of the grain remained intact. Grain was stored aseptically at 4 °C until used.

### **2.2.1 Modification of media and substrates water activity levels**

The water activity ( $a_w$ ) of agar-based media was adjusted by the addition of glycerol as determined by Dallyn (1980). Wheat grain  $a_w$  levels were adjusted by the addition of sterile distilled water. The quantity of water required was determined by using a moisture absorption isotherm which was constructed for every batch of grain used in the experiments. An Aqualab Series 3 (Labcell Ltd., Basingstoke, Hants, UK) was used to measure the  $a_w$  levels of the substrates prior to use, and throughout the experiments. The  $a_w$  levels were kept constant throughout by keeping the same  $a_w$  treatments in airtight containers. In the case of the grain substrates a beaker of glycerol/water solution of the same  $a_w$  as the experimental treatment was also included inside the container to humidify the internal atmosphere to the appropriate level.

## **2.3 IN VITRO COMPARISONS OF ENVIRONMENTAL CONDITIONS ON THE GROWTH OF *F. CULMORUM* AND COMMON WHEAT SPOILAGE FUNGI**

The aim of this experiment was to determine how varying  $a_w$  and temperature conditions had on the growth rates of various *F. culmorum* isolates from different regions of Europe. Wheat agar adjusted to 0.995  $a_w$ , 0.98  $a_w$ , 0.95  $a_w$  and 0.93  $a_w$  was prepared and poured into 90 mm Petri dishes. Plates were inoculated with 1  $\mu\text{l}$   $10^5$  spore suspension of the *F. culmorum* strains :- UK1, UK2, UK3, ITA1, ITA2, ITA3, NOR1 and NOR2. Inoculated plates were then incubated at either 15 °C or 25 °C.



Colony diametric measurements were made frequently throughout the incubation period by taking two readings at right angles to each other. Data was then tabulated and the linear portion of the radial extension rates used to determine growth rate via linear regression.

## **2.4 TWO DIMENSIONAL ENVIRONMENTAL PROFILES OF GROWTH AND MYCOTOXIN PRODUCTION BY *F. CULMORUM* AND *F. GRAMINEARUM* ON WHEAT AGAR AND IRRADIATED WHEAT GRAIN**

The aim of this study was to provide detailed information on growth of *F. culmorum* and *F. graminearum* and mycotoxin production across a wider range of  $a_w$  conditions than tested previously. Also, samples were taken at different time intervals and analysed for mycotoxins. This study enabled information to be gathered on optimum and minimum growth and mycotoxin production conditions for both these species.

Wheat agar and irradiated wheat grain Petri plate cultures were prepared at (0.99-0.85  $a_w$ ). 24 plates of each  $a_w$  treatment and substrate were inoculated centrally with a  $10^5$  ml<sup>-1</sup> *F. culmorum* or *F. graminearum* macroconidial suspension obtained from a seven day old colony. Plates of the same  $a_w$  were enclosed in plastic bags, sealed and incubated at 15 or 25 °C, for up to 40 days. Temporal growth measurements were taken throughout the incubation period, by taking two diametric measurements of the colonies at right angles to each other. Growth rates were determined subsequently by linear regression of the linear growth phase. Three replicates per treatment were removed after 10, 20, 30 or 40 days and analysed for the mycotoxins DON and NIV using the method detailed in section 2.12 .



## 2.5 INTERSPECIFIC INTERACTIONS BETWEEN GRAIN FUNGI ON AGAR AND IRRADIATED WHEAT GRAIN AND THE EFFECT ON DON AND NIV PRODUCTION

The aims of this study were to:

- a. Determine how *F. culmorum* interacts with a number of other wheat grain niche fungi on wheat agar and irradiated wheat grain substrates under various temperatures and  $a_w$  levels.
- b. Investigate the effects of their interactions under different environmental stresses on DON and NIV production by *F. culmorum*.

Wheat agar adjusted to 0.995  $a_w$ , 0.980  $a_w$  and 0.955  $a_w$  was prepared. Spore suspensions ( $10^5 \text{ ml}^{-1}$ ) were prepared from seven day old colonies of *A. tenuissima*, *C. herbarum*, *F. culmorum* (UK3), *F. graminearum*, *F. poae*, *M. nivale* var. *majus* (1/1/M), *M. nivale* (18/1/N) and *P. verrucosum*. Treatment plates were inoculated with *F. culmorum* and one of the other species. Plates were inoculated using a 1  $\mu\text{l}$  calibrated loop and the inoculation point for each species was approximately 4 cm apart. The controls were homogenous cultures inoculated with a single point inoculation using the 1  $\mu\text{l}$  loop. Plates of the same  $a_w$  were sealed in plastic bags and incubated at 15 or 25 °C. Each treatment and condition was carried out in triplicate. Irradiated wheat grain plates followed a similar methodology to the wheat agar plates, the only differences being the exclusion of the 0.98  $a_w$  treatment and the incubation of plates in chambers humidified to the same  $a_w$  as the plates. The plates were incubated for 30 days and then analysed for mycotoxins.

During the incubation period, colony diameter was measured by taking two measurements at right angles to each other. The interactions between mycelia of dual cultures were determined by macroscopic and microscopic analysis. Based on these observations each interaction was given a score based on mutual intermingling (1-1), mutual antagonism on contact (2-2), mutual antagonism at a distance (3-3), dominance of one species on contact (4-0) and dominance at a distance (5-0). In the case of the dominant interactions the higher score is always awarded to the more



competitive fungus (Magan & Lacey, 1984a). For instance if *F. culmorum* was dominant over *F. graminearum* upon contact this would result in a 4 and 0 respectively being awarded to the two fungial species. Scores were totalled to obtain an overall Index of Dominancy ( $I_D$ ) value as a measure of competitiveness.

## 2.6 CARBON SOURCE UTILIZATION BY *F. CULMORUM* AND OTHER COMMON WHEAT SPOILAGE FUNGI (NICHE OVERLAP INDEX)

The aim of these studies were to investigate how nutritional utilization patterns and niche overlap index of *F. culmorum* and other wheat grain fungi change with fluctuating  $a_w$  and temperatures. This provided insight as to why some species are more competitive/dominant than others under certain conditions.

Biolog GN plates (Biolog, Inc. CA, USA) were used to assay the nutrient utilisation of the fungi. The Biolog GN plate is a 96 well microtitre plate. It contains 95 different carbon sources including: carbohydrates, carboic acids, amino acids, amines and amides. The most important carbon sources present in the Biolog plates for wheat grain niche fungi were: Dextrin, D-fructose, D-galactose,  $\alpha$ -D-glucose, D-melobiose, D-raffinose, sucrose, D-alanine, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-phenylalanine, L-proline, D-serine, L-serine and L-threonine.

Spore suspensions were prepared from 7 day old colonies from the following species: *F. culmorum*, *F. poae*, *F. graminearum*, *P. verrucosum*, *M. nivale* var. *majus*, *C. herbarum*, and *A. tenuissima*. These spore suspensions were washed 3 times to remove any remaining media, in 10ml of 0.25M 2-(N-morpholino) ethanosulphonic acid (MES, Sigma) buffered to pH 5.5. MES modified to 0.995  $a_w$  and 0.93  $a_w$  with NaCl was used to make spore suspensions ( $10^6 \text{ ml}^{-1}$ ) of each of the fungal treatments. The Biolog plates were inoculated by pipetting 100 $\mu$ l of spore suspension into each well. The plates were covered and replicats of the same  $a_w$  levels placed into sealed containers equilibrated to the desired  $a_w$  with a 400ml beaker of glycerol/water solution of the  $a_w$  to maintain ambient conditions. Plates were incubated at 15 or 25 °C for 14 days. Qualitative determination of growth within the wells of the Biolog



plates was done via microscopic analysis. Niche overlap index (NOI) was calculated by dividing the number of C sources in common between two fungi and the total number of C sources utilized by the test organism. In this case the test organism was *F. culmorum*. Using this system NOI of <0.90 is indicative of occupation of separate niches, and >0.90 of shared niches (Magan & Lacey, 1984a; Marin *et al.*, 1997a; Lee & Magan, 1999a; Lee & Magan, 2000).

## **2.7 ENZYME PRODUCTION BY WHEAT GRAIN NICHE FUNGI AT DIFFERENT $A_w$ AND TEMPERATURES ON IRRADIATED WHEAT GRAIN**

Enzyme production and activity by various wheat grain fungi may be indicative of relative capacity for deterioration of grain under different abiotic factors. It was also hoped that this would provide some information on why some species were dominant under certain environmental conditions.

Irradiated wheat grain adjusted to 0.99, 0.95 and 0.93  $a_w$  was placed in 90 mm Petri dishes to form a monolayer of wheat grain. Spore suspensions of  $10^6$  ml<sup>-1</sup> were prepared from seven day old colonies of the following species: *A. tenuissima*, *C. herbarum*, *F. culmorum*, *F. poae*, *F. graminearum*, *M. nivale* var. *majus* and *P. verrucosum*. 100 µl of the spore suspension was used to inoculate the irradiated wheat grain. Plates were shaken by hand to distribute the inoculum throughout the grain. Plates of the same  $a_w$  were placed into containers, closed and incubated at 15 or 25 °C. The plates were incubated for 7, 14 and 21 days after which they were removed for enzyme extraction. All enzyme extraction was carried out within 8 hours. All treatments were replicated three times.



### 2.7.1 Enzyme extraction

Enzymes were extracted by weighing 2 g of sample into Universal bottles containing 4 ml of 10mM potassium phosphate buffer pH 7.2. These were then shaken for 1 hour on a rotary shaker at 4 °C. The washings were transferred to 1.5 ml Eppendorfs tubes and then centrifuged at high speed in a microfuge to pellet any suspended solids. The supernatant was carefully removed and placed in -80 °C storage for subsequent enzyme analysis.

### 2.7.2 Total enzyme analysis

Total enzyme analysis determines enzyme activity based on the abilities of the enzymes to liberate *p*-nitrophenol via enzymatic hydrolysis of *p*-nitrophenol substrates. Seven *p*-nitrophenol substrates were used to assay the activity of their corresponding glycosidases. The substrates and their buffers are listed in Table 2.2. The assay was performed using 96 well microtitre plates allowing multiple assays to be carried out at the same time. The reaction mixture pipetted into each test well consisted of the following: enzyme extract (40  $\mu$ l), substrate solution (40  $\mu$ l) and the appropriate buffer (20  $\mu$ l). The microtitre plates were then covered and incubated at 37 °C for 1 hour. The reaction was stopped using 5  $\mu$ l of 1M Na<sub>2</sub>CO<sub>3</sub> and the plates allowed to cool for 3 min before being read. The control wells contained enzyme extract and buffer prior to incubation. After the stop agent had been added, the substrate solution was added to the control wells. Plates were analysed using a MRX multiscan plate reader (Dynex Technologies Ltd, Billingham, West Sussex, UK) using a 405 nm filter. Total enzyme activity was calculated by comparison with a calibration curve of absorbance at 405 nm against *p*-nitrophenol concentration. Total enzyme activity is expressed as  $\mu$ mol *p*-nitrophenol released min<sup>-1</sup>.



### 2.7.3 Determining total protein and specific enzyme activities

Total protein content of the enzyme extracts was determined by using the Bicinchoninic Acid Protein Assay Kit (Sigma). The kit contains bicinchoninic acid solution, copper (II) sulphate pentahydrate 4 % solution and a protein standard (BSA 1.0mg ml<sup>-1</sup>). The assay is colourmetric, utilizing the abilities of proteins to reduce alkaline Cu(II) to Cu(I), which forms a purple complex with bicinchoninic acid. Absorbance of the resulting solution at 550 nm is directly related to protein concentrations.

The bicinchoninic acid and the Cu(II) solution are combined at a 50:1 ratio for the working reagent. This reagent is stable for one day at room temperature. A 10  $\mu$ l aliquot of a sample was placed in the appropriate microtitre plate wells. In the blank wells 10  $\mu$ l of potassium phosphate buffer (10mM pH 7.2) was used in place of the samples. Using a multi-channel pipette, 200  $\mu$ l of working reagent was placed in all of the wells. Plates were covered and shaken. The plates were then incubated at 37 °C for 30 min. The plates were allowed to cool after being removed from the incubator, the absorbance at 550 nm was then determined using a plate reader. Protein concentration in the enzyme extract samples was determined by extrapolation from a calibration curve made with the BSA standards. These results in combination with the total enzyme activity allow the specific enzyme activity to be determined, which is expressed as nmol p-nitrophenol released min<sup>-1</sup>  $\mu$ g<sup>-1</sup> protein.

## 2.8 EFFICACY OF COMMON FUNGICIDES ON GROWTH OF *F. CULMORUM* IN VITRO

Previous experiments established how naturally occurring biotic and abiotic factors affect *F. culmorum*. The aim of this experiment was to determine how common fungicides affect the growth of *F. culmorum* under different temperatures and  $a_w$  levels. The fungicides used and their percentage active ingredient are shown in Table 2.3. Wheat agar was adjusted to 0.995  $a_w$ , 0.985  $a_w$  and 0.955  $a_w$ . Prior to pouring, after the media had cooled to approximately 50 °C, the fungicides were incorporated



**Table 2.2.** Summary of the enzyme assay, associated substrate, concentration, buffer and pH.

Enzyme	Substrate	Conc (mM)	Buffer	pH
$\beta$ -D-fucosidase	4-nitrophenyl- $\beta$ -D-fucopyranoside	2.0	25 mM acetate	5.0
$\alpha$ -D-galactosidase	4-nitrophenyl- $\alpha$ -D-galactopyranoside	4.0	25 mM acetate	5.0
$\beta$ -D-glucosidase	4-nitrophenyl - $\beta$ -D-glucopyranoside	2.0	25 mM acetate	5.0
$\alpha$ -D-mannosidase	4-nitrophenyl - $\alpha$ -D-mannopyranoside	4.0	25 mM acetate	5.0
$\beta$ -D-xylosidase	4-nitrophenyl- $\beta$ -D-xylopyranoside	2.0	25 mM acetate	5.0
<i>N</i> -acetyl- $\alpha$ -D-glucosaminidase	p-nitrophenyl- <i>N</i> -acetyl- $\alpha$ -D-glucosaminide	2.0	25 mM acetate	4.2
<i>N</i> -acetyl- $\beta$ -D-glucosaminidase	p-nitrophenyl- <i>N</i> -acetyl- $\beta$ -D-glucosaminide	2.0	25 mM acetate	4.2

**Table 2.3** List of fungicides, their commercial name, active ingredient and company. These fungicides were used in experiments 2.8 and 2.9 .

Fungicide	Active ingredient	Company
Amistar	Azoxystrobin 250g l <sup>-1</sup>	Zeneca
Tilt	Propioconazole 250g l <sup>-1</sup>	Ciba-Geigy
Opus	Epoxiconazole 125g l <sup>-1</sup>	BASF



into the media to obtain 0, 1, 5, 25, 50  $\mu\text{g ml}^{-1}$  of fungicide active ingredient. The molten medium was then poured into 90mm Petri dishes. A loop of  $10^6$  conidial suspension from a seven day old *F. culmorum* (UK3) culture was used to inoculate the wheat agar plates. Plates of the same  $a_w$  were sealed in plastic bags and incubated at 15 and 25 °C. Diametric size of the colonies was taken at regular intervals during the incubation period by taking two measurements at right angles to each other. The experiment was replicated in triplicate.

## **2.9 INTERACTIONS BETWEEN NICHE FUNGI IN THE PRESENCE OF SUB-OPTIMAL LEVELS OF COMMON FUNGICIDES ON AGAR AND IRRADIATED WHEAT GRAIN AND EFFECTS ON DON AND NIV PRODUCTION**

Building upon the previous interaction experiments this study included the introduction of fungicides as an additional stress factor. The aim of this experiment was to determine how the presence of suboptimal concentrations of commercial fungicides affect interspecies interactions and mycotoxin production. Wheat agar was adjusted to 0.995, 0.98 and 0.955  $a_w$ . Prior to pouring, after the media had cooled to approximately 50 °C, the fungicides (Table 2.3) were incorporated into the media to result in 0.5  $\mu\text{g ml}^{-1}$  of fungicide active ingredient. Spore suspensions  $10^5 \text{ ml}^{-1}$  were prepared from seven day old colonies of *A. tenuissima*, *C. herbarum*, *F. culmorum* (UK3), *F. graminearum*, *F. poae*, *M. nivale* var. *majus* (1/1/M), *M. nivale* (18/1/N) and *P. verrucosum*. Treatment plates were inoculated with *F. culmorum* and one of the other isolates. These plates were inoculated using a 1  $\mu\text{l}$  calibrated loop and the inoculation point for each species was approximately 4 cm apart. The controls were homogenous cultures inoculated with a single point inoculation using 1  $\mu\text{l}$  loop. Plates of the same  $a_w$  treatment were sealed in plastic bags and incubated at 15 or 25 °C. Each treatment and condition was carried out in triplicate.

Irradiated wheat grain plates followed a similar methodology to the wheat agar plates, the only differences being the exclusion of the 0.98  $a_w$  condition and the incubation of plates in chambers humidified to the same  $a_w$  as the plates. Fungicides were incorporated into the irradiated wheat grain substrate by adding the fungicide to the



water used to adjust the grain  $a_w$ . This ensured that the fungicides were evenly distributed throughout the substrate. The plates were incubated for 30 days and then analysed for mycotoxins using the method detailed in section 2.12 .

## **2.10 THE EFFICACY OF ESSENTIAL OILS AND ANTIOXIDANTS AS CONTROL TREATMENTS FOR GROWTH AND TOXIN PRODUCTION OF COMMON ORGANISMS RESPONSIBLE FOR CAUSING *FUSARIUM* EAR BLIGHT**

The aim of this study was to determine if essential oils or antioxidants could be used to control growth and mycotoxin production by *Fusarium* species responsible for wheat grain infection.

The *in vitro* screen was a two-stage process. A wide spectrum initial screen, which identified promising control agents, was first carried out. The essential oils and antioxidants that were able to significantly inhibit the *Fusaria* in the initial screen were then studied in more detail *in vitro* and then *in situ*. The early stages of the screening process (sections 2.10.1 , and 2.10.2 ) were only concerned with the effects of the essential oils and antioxidants on growth. In the *in situ* study (section 2.10.3 ) on irradiated wheat grain the effect of essential oils and antioxidants on growth and toxin production was considered.

### **2.10.1 Initial essential oil and antioxidant screen**

Two techniques were used in the initial screening of 23 essential oils (basil, bay leaf, lime, rosemary, cinnamon leaf, pine sylventris, orange, peppermint yakima, ginger, marjoram, eucalyptus, clove, lemongrass, grapefruit, sweet fennel, mandarin red, lemon sicilian, thyme, basil linalol, aniseed, nutmeg, spearmint and sage (F.D. Copeland & Sons, Ltd., London) and six antioxidants (propyl gallate, propyl paraben (PP), butylated hydroxy anisole (BHA), propionic acid, benzoic acid and butylated hydroxytoluene (BHT, Sigma).



The initial essential oil screen was a zone of inhibition bioassay and the antioxidant initial screen was by measurement of effects on temporal growth. The basic medium for both methods was a 2 % milled wheat agar. The  $a_w$  of the agar was modified to 0.995  $a_w$  by the addition of glycerol (Dallyn & Fox, 1980). The inoculum used was a *F. culmorum* spore suspension at  $10^6$  ml<sup>-1</sup>, obtained from 7 day old plate cultures (MEA). The results from the initial screen demonstrated that only three essential oils and two antioxidants had potential for control of *F. culmorum*.

### **2.10.2 Efficacy of essential oils and antioxidants on fungal growth on agar**

A detailed temporal study of the efficacy of clove, bay and cinnamon oils and the antioxidants BHA and propyl paraben were screened at 25, 50, 100, 200, 500 and 1000 ppm on a 2 % milled wheat agar at three  $a_w$  levels (0.995, 0.985 and 0.955  $a_w$ ) at 15 and 25 °C. The essential oils and antioxidants were dissolved in 5 ml of methanol and then added to the molten media to produce the final concentrations. The control was the basic medium to which 5 ml of methanol only was added. Plates were inoculated centrally with  $10^6$  spores ml<sup>-1</sup> of a macroconidial suspension of *F. culmorum*, *F. graminearum* or *F. poae* obtained from a 7 day old plate culture (MEA) using a 1 µl sterile loop. Plates of the same  $a_w$  were sealed in plastic bags and incubated at 15 or 25 °C. Colony diameter was measured at regular intervals by taking two readings at right angles to each other. Experiments were carried out with two replicates of all treatments and repeated once.

### **2.10.3 Efficacy of essential oils and antioxidants at controlling growth and mycotoxin production of *Fusarium* species on an irradiated wheat grain substrate.**

This experiment used essential oils and antioxidants identified as being able to inhibit *F. culmorum* growth in the initial screen (section 2.10.1 ). Irradiated wheat grain was weighed and placed in sterile flasks. Essential oil or antioxidant was dissolved in 5 ml of methanol to result in an end concentration of either 100 or 500 ppm in the grain (w/w), with the control treated with methanol only. This solution was then



incorporated into the sterile water to be used for the  $a_w$  adjustment to 0.995  $a_w$  and 0.955  $a_w$ . This was then added to the flasks. The flasks were allowed to equilibrate at 4 °C for 96 hours, with periodic shaking. 24 hours before inoculation, the grain from the flasks was poured into Petri plates to obtain a monolayer of wheat grain of approximately 20 g of grain in each plate.

Plates were inoculated centrally with a loop of  $10^6$  ml<sup>-1</sup> spore suspension of the appropriate *Fusarium* species. Inoculated plates of the same  $a_w$  were then placed in a sealed container including a beaker with 250 ml of a water/glycerol solution at the same  $a_w$  as the treatment to help maintain equilibrium relative humidity of the chamber. Four replicates per treatment were incubated at 15 and 25 °C for 30 days. The colony diameters were measured daily or as required for the 30 day incubation period.

At the end of the incubation period the samples were analysed for trichothecenes. The Department of Chemistry at the National Veterinary and Food Research Institute in Helsinki carried out trycothecene analysis using Gas Chromatograph-Mass Spectrometry method that had been optimised for the substrate type (Eskola *et al.*, 2001; Eskola & Rizzo, 2002).

## **2.11 EFFECT OF ESSENTIAL OILS AND ANTIOXIDANTS ON THE NATURAL FUNGAL POPULATIONS AND THE LEVELS OF DON AND NIV IN GRAIN AT DIFFERENT ENVIRONMENTAL CONDITIONS**

The aim of this experiment was to determine the effect the essential oils and antioxidants identified by the previous study (section 2.10 ) had on naturally contaminated wheat grain. This was achieved by an *in situ* study to determine efficacy of specific essential oils and antioxidants on the inhibition of fungi and trichothecene production on non-sterilised wheat grain spiked with *F. culmorum*. Three essential oils and two antioxidants were used as grain treatments (essential oils; clove, bay and cinnamon, antioxidants; BHA and propyl paraben). The *F. culmorum* isolate used in this experiment was the same as used in the previous studies, *F. culmorum* UK3.



Non-sterile wheat grain was weighed and placed in sterile flasks. Essential oils and antioxidants were dissolved in 10 ml of methanol to result in an end concentration of  $200\mu\text{g g}^{-1}$  in the grain (control treatment was methanol alone). This solution was then incorporated into the sterile water to be used for the  $a_w$  adjustment (0.93, 0.955 and 0.97). This was then added to the flasks. The flasks were allowed to equilibrate at 4 °C for 96 hours, with periodic shaking. The grain was then distributed in 100 g portions into solid culture vessels (Magenta; Sigma). The grain was inoculated with 1 ml of  $10^4$  microconidial suspension of *F. culmorum* obtained from a 7 day old colony grown on MEA. The culture vessels were shaken by hand to distribute the inoculum throughout the media. Samples of the same  $a_w$  level were placed in sealed chambers, humidified to the same  $a_w$  level with glycerol/water solutions. Samples were incubated at 15 or 25 °C for a 28 day period. Every seven days a 20 g sub-sample was taken from each culture vessel. One gram of the sub-sample was used for determining fungal populations, including *Fusarium* species colony forming units (CFU) using serial dilutions and direct plating of grain and the remainder frozen for subsequent DON/NIV analysis.

For the serial dilution one gram of grain was shaken in 9mls of sterile water/tween 80 solution and a serial dilution series was performed to produce concentrations of  $10^{-1}$  to  $10^{-5}$ . 100  $\mu\text{l}$  of each dilution was spread-plate onto MEA plates adjusted to the same  $a_w$  level as the original samples. Plates of the same  $a_w$  were sealed in plastic bags and incubated at the same temperature as the original samples. For the direct plates 10 grains of each sample were placed equal distances apart on MEA plates adjusted to the same  $a_w$  as the original sample and incubated at the same temperature as the original. After 5-7 days the colonies were counted and where possible identified to the genus level.

## **2.12 HPLC ANALYSIS FOR THE DETECTION OF DON AND NIV**

Mycotoxin extraction was adapted from (Cooney *et al.*, 2001). Agar and the mycelium from each sample were placed in 40 ml of acetonitrile/methanol (14:1) for 12 hours. 2 ml was taken for DON and NIV analysis and passed through a cleanup cartridge. The



cleanup cartridges (Figure 2.1) were made in-house specifically for the workup of the samples. Each cartridge consisted of a 2 ml syringe (Fisher Ltd.) packed with a disc of filter paper (No1 Whatman International Ltd.), a 5 ml lugger of glass wool and 300 mg of alumina/activated carbon (20:1). The sample was allowed to gravity feed through the cartridge. Residues on the cartridge were washed out with acetonitrile/methanol/water (80:5:15; 500  $\mu$ l). The combined eluate was evaporated (compressed air, 50 °C) and then re-suspended in methanol/water (5:95; 500  $\mu$ l). Percentage recovery of DON with this method was 88 %  $\pm$ 2.6 %, and for NIV 85 %  $\pm$ 3.2 %.

Quantification of DON and NIV was accomplished via HPLC, using a Luna column (100mm x 4.6mm i.d.) (Phenomenex). Separation was achieved using an isocratic mobile phase of methanol/water (12:88) at 1.5 ml min<sup>-1</sup>. Eluates were detected using a UV detector set at 220 nm and an attenuation of 0.01 AUFS. The retention times for NIV and DON were 3.4 and 7.5 minutes respectively. Concentrations of NIV and DON were determined by extrapolation from a standard curve (Figure 2.2) constructed from external standards injected at the beginning of every HPLC sample batch run. Limit of quantification for DON 120 ng ml<sup>-1</sup> and 100 ng ml<sup>-1</sup> for NIV.

### **2.12.1 Changes in method for DON and NIV extraction from a wheat grain matrix**

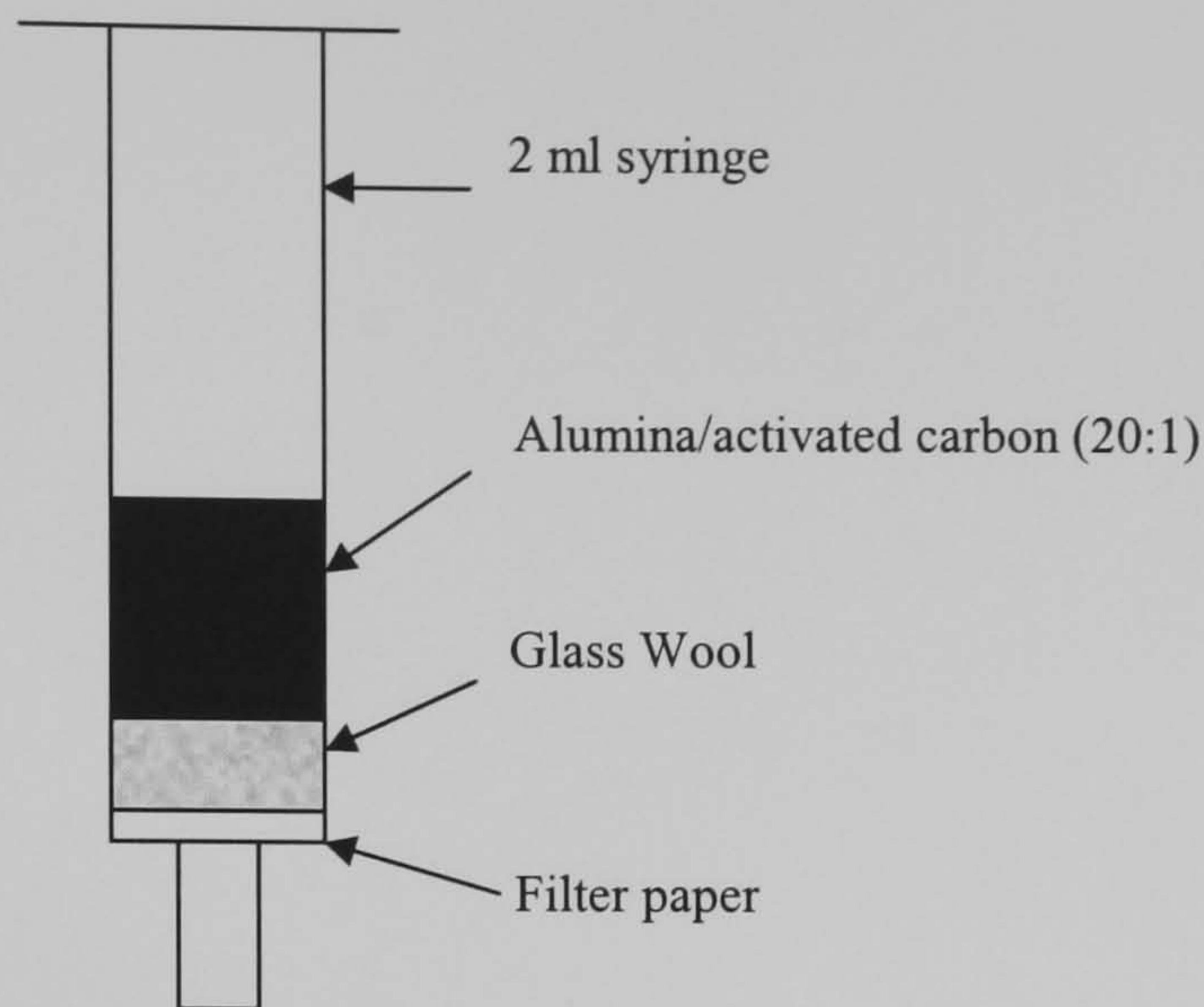
The following alterations were made to the above extraction and clean up method when dealing with wheat grain samples. Samples were first dried for 24 hours below 50 °C (approx 48 °C). The dry samples were then milled. Ten grams of the milled sample was then placed into 40 ml of acetonitrile/methanol (14:1). This solution was shaken vigorously for 2 hours on a rotary shaker and then filtered (No1 Whatman International Ltd). 2 ml of this solution was passed through a cleanup cartridge (Figure 2.1) similar to those used for agar extraction. The only difference was an increase in the quantity of alumina/activated carbon packing to 500 mg. The remaining steps were the same as those used for agar samples.



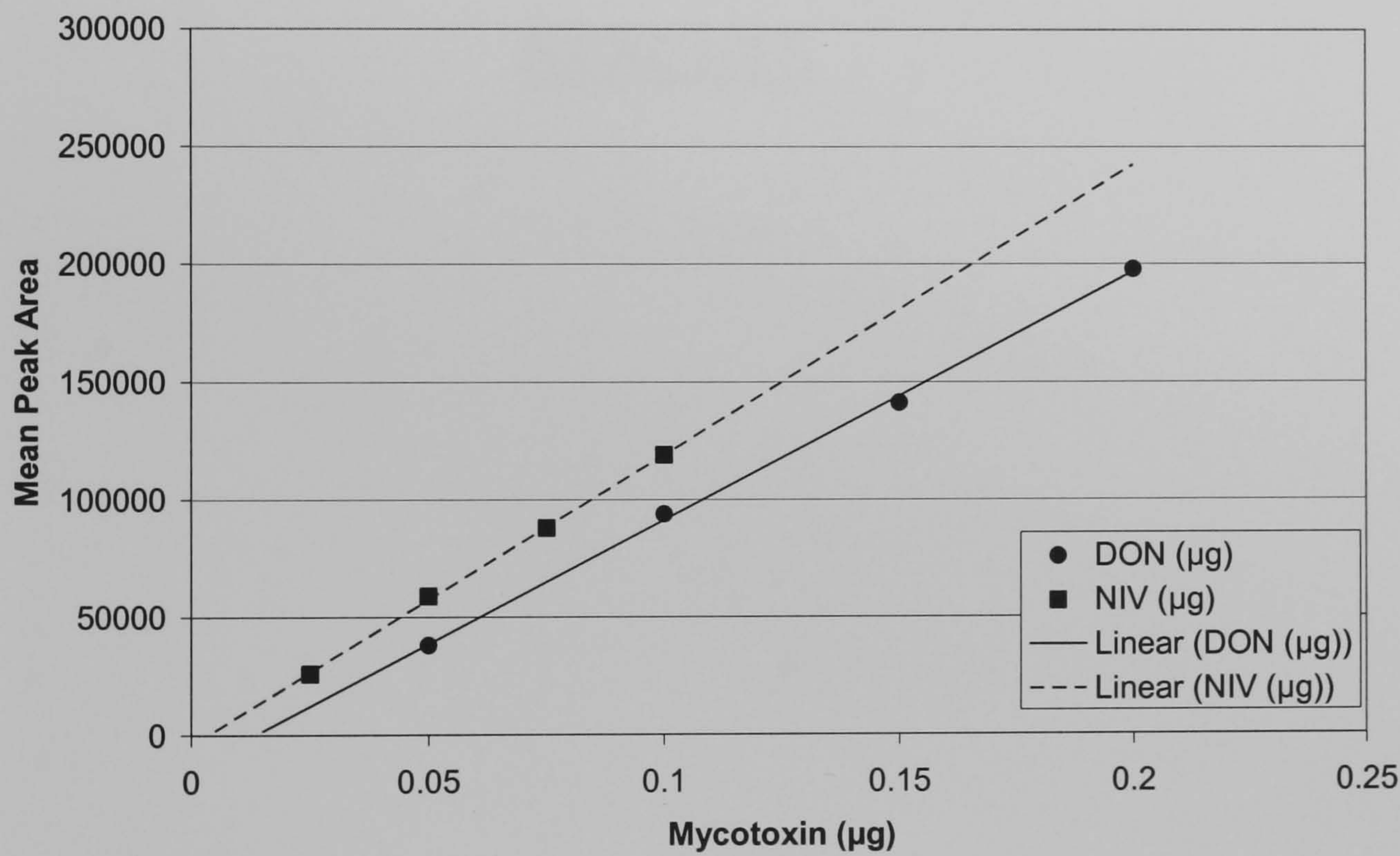
## 2.13 STATISTICAL HANDLING OF DATA

Data input, data handling/manipulation, linear regression, and graph plotting was carried out using Microsoft Excel 2002 (Microsoft Co.). Other statistical tests (i.e. ANOVA and mean effect plots) were performed using Minitab 13.32 (Minitab inc.). For ANOVA and LSD significance was deemed to be when  $P = <0.05$ . Unless otherwise stated in the text ANOVA tables can be found in Appendix I, Section I.II.





**Figure 2.1** Cross-section of in house clean up cartridge.



**Figure 2.2** Example of a calibration for DON and NIV generated from the external standards at the beginning of a HPLC run.



# CHAPTER 3

## RESULTS



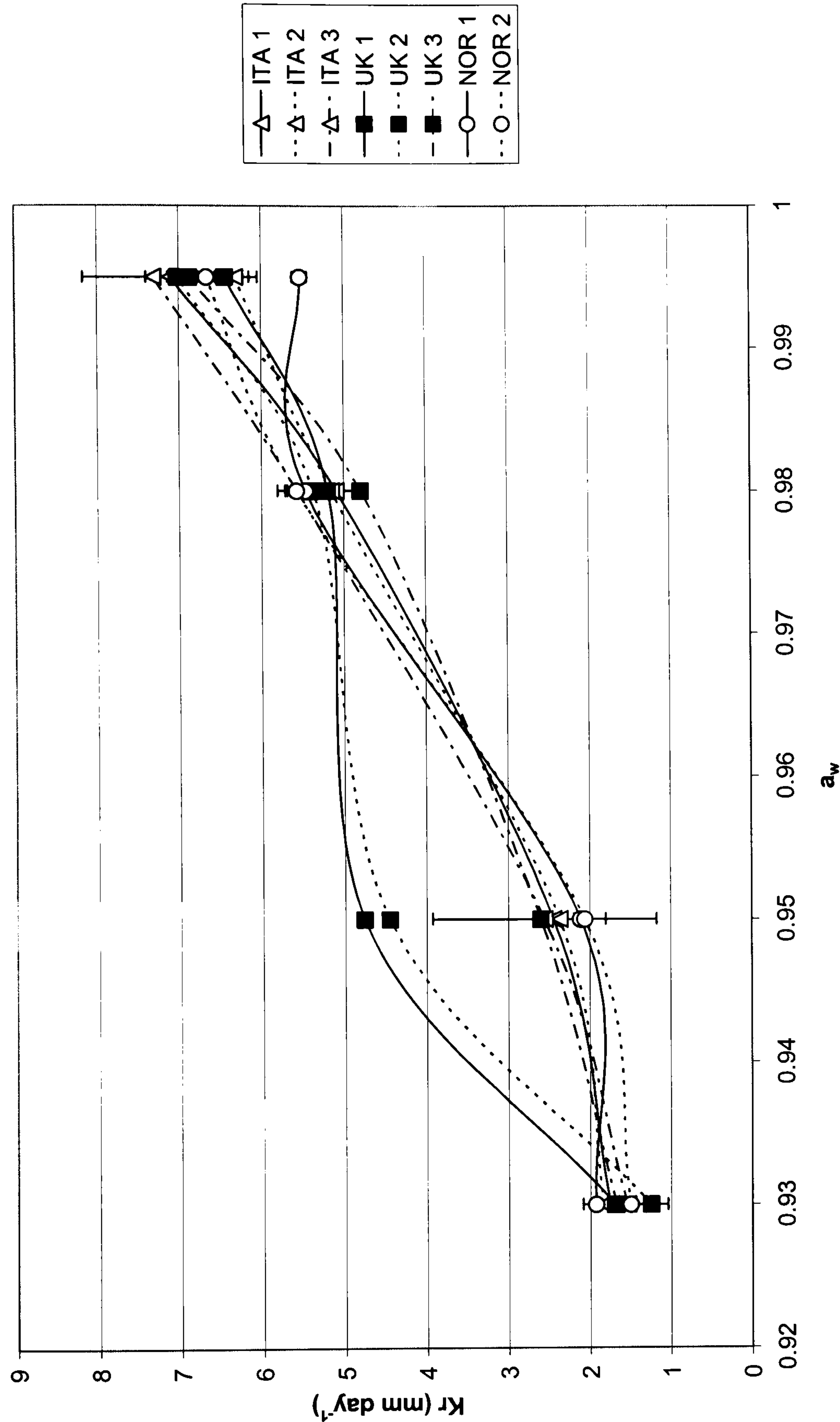
### 3.1 COMPARISON OF THE GROWTH RATES OF *F. CULMORUM* ISOLATES ORIGINATING FROM DIFFERENT REGIONS OF EUROPE

Figures 3.1 and 3.2 show the growth rates obtained for different isolates of *F. culmorum* under various  $a_w$  levels at 15 and 25 °C respectively. The different isolates originate from various countries within Europe.

The  $a_w$  levels had a dramatic effect on growth of all the isolates. Growth rates generally increased as  $a_w$  levels increased. However, all the isolates apart from UK3 produced their highest rate of expansion at 0.98  $a_w$  and 25 °C. This was not seen at 15 °C where optimum growth rate were observed when water was freely available (0.995 $a_w$ ). Temperature had a smaller impact on growth rates than  $a_w$  level. Growth rates at 15 °C were generally lower than those at 25 °C. However, this temperature effect on growth rate diminished as  $a_w$  level was reduced. For instance growth rates at 0.93  $a_w$  were almost identical at either 15 °C or 25 °C. The different isolates generally reacted in a similar way to the various levels of  $a_w$  and temperature. The exception to this was UK3 which was significantly more sensitive to  $a_w$  changes at 25 °C than the other isolates. This resulted in UK3 having much lower growth rates at 0.98  $a_w$  at this temperature than the other isolates.

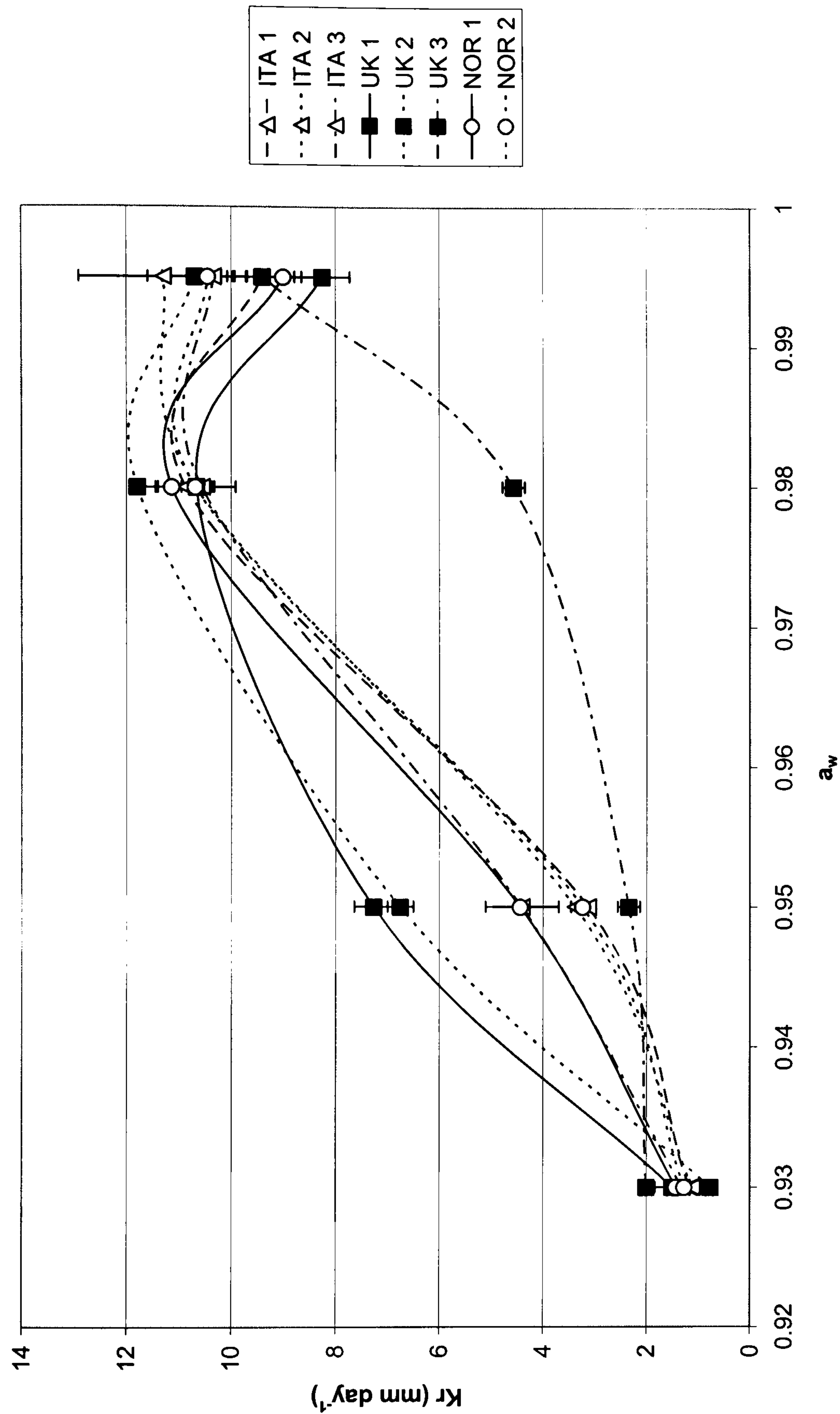
Mycelium produced by all the isolates was floccus at the higher  $a_w$  levels. At 0.95  $a_w$  and below, aerial mycelium was only sparsely produced. Colonies were heavily pigmented after three to four days incubation, colours varied from golden brown to deep reds, differing slightly from isolate to isolate and dependant on the  $a_w$  and temperature conditions.





**Figure 3.1** Growth of *F. culmorum* strains from different regions of Europe on wheat agar adjusted to various  $a_w$  levels and incubated at 15 °C. Bars indicate standard deviation.





**Figure 3.2** Growth of *F. culmorum* strains from different regions of Europe on wheat agar adjusted to various  $a_w$  levels and incubated at 25 °C. Bars indicate standard deviation.



## 3.2 TWO DIMENSIONAL ENVIRONMENTAL PROFILES OF GROWTH AND MYCOTOXIN PRODUCTION BY *F. CULMORUM* AND *F. GRAMINEARUM* ON WHEAT AGAR AND IRRADIATED WHEAT GRAIN

If *F. culmorum* and *F. graminearum* are to be effectively controlled, then information on how their growth and mycotoxin production are affected by the different conditions present in the environment where control is required (i.e. during ripening preharvest and during drying of stored wheat) is of key importance. Examples of when this type of information is important are: determining critical control points in HACCP schemes and the timing of control treatment applications pre- and post-harvest.

### 3.2.1 Temperature x $a_w$ interaction effects on growth and DON/NIV production by *F. culmorum* on wheat agar

Figure 3.3 shows the radial extension rates ( $K_r$ ) determined for *F. culmorum* on wheat agar at the  $a_w$  levels and temperatures studied. The highest  $K_r$  was produced at 25 °C and 0.995  $a_w$ . There was no mycelial growth observed at  $\leq 0.90$   $a_w$ . Although no growth was seen at the lower  $a_w$  levels, zones of clearing were produced in the opaque agar around the inoculation point. Growth rates decreased as  $a_w$  was lowered. Temperature generally affected growth, with mycelial extension at 25 °C being higher than at 15 °C. Temperature effects were more marked at 0.995  $a_w$ . At lower  $a_w$  levels, temperature only elicited a small effect on growth rates. Colonies consisted of dense heavily pigmented mycelium at 25 °C and at the higher  $a_w$  levels. At  $\leq 0.96$   $a_w$  and below mycelium was diffuse and had little pigmentation. Statistical analysis (Table 3.1) shows that  $a_w$  temperature and their interactions significantly affected growth.

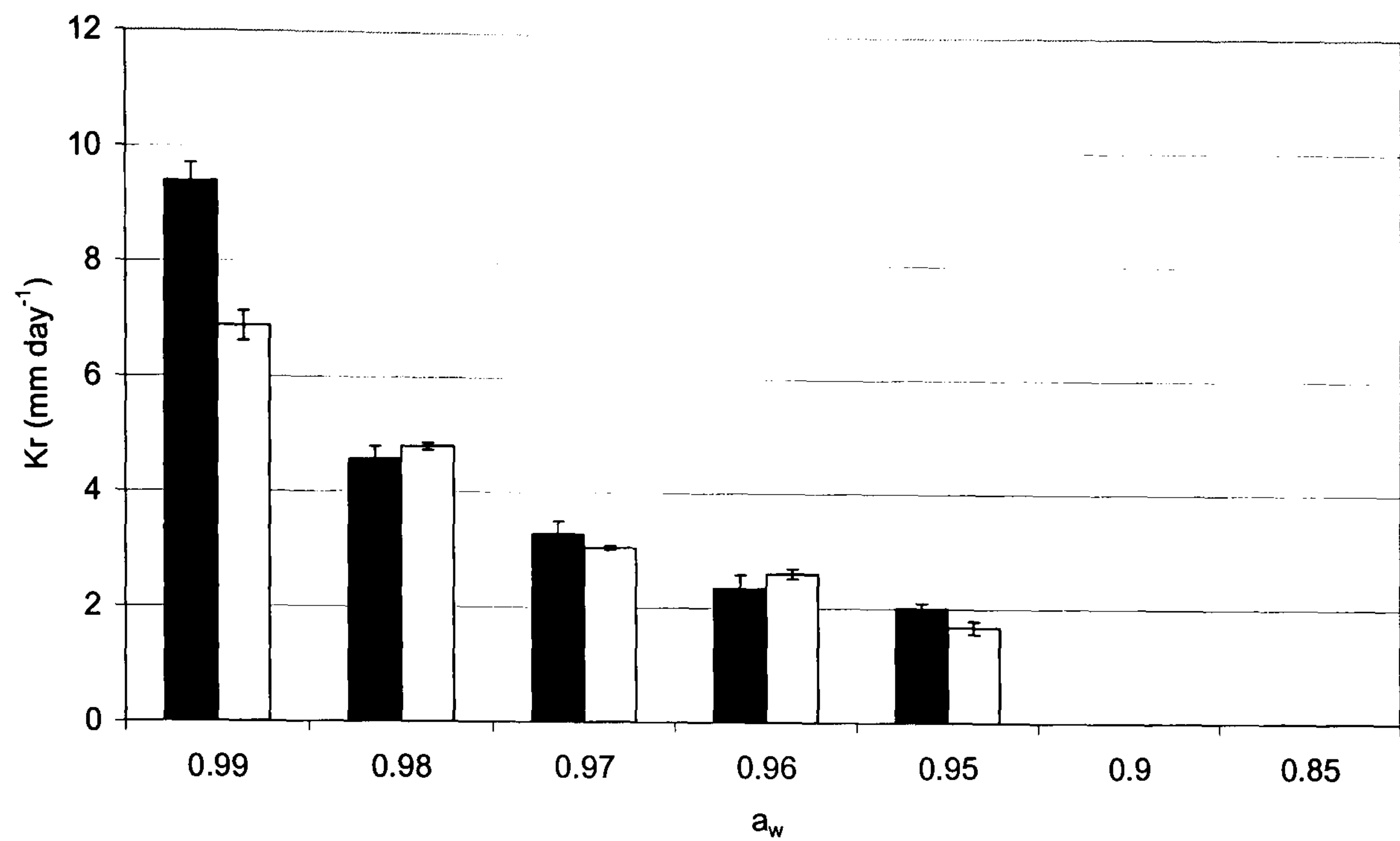
Figures 3.4 and 3.5, shows the temporal changes in DON levels determined for the different conditions. The highest DON levels were obtained at 0.995  $a_w$ , 25 °C after 40 days incubation. As  $a_w$  levels were reduced DON production rapidly declined. DON production was lower at 15 °C than at 25 °C and generally increased as



incubation time increased. The effects of  $a_w$ , temperature and incubation time and their interactions was found to be significant (Table 3.2).

Figure 3.6 and 3.7 show the NIV levels determined for the different conditions and incubation times. The highest NIV levels were obtained at 0.98  $a_w$ , 25 °C after 40 days incubation. NIV production was approximately 10 times less than DON at the higher  $a_w$  levels and 25 °C. The factors  $a_w$ , temperature and incubation time had a significant effect on NIV production (Table 3.3). The interactions between  $a_w$  x temperature and temperature x incubation time were also found to be of significance. Incubation time x  $a_w$  and  $a_w$  x temperature x incubation time interactions did not have a significant effect on NIV production.



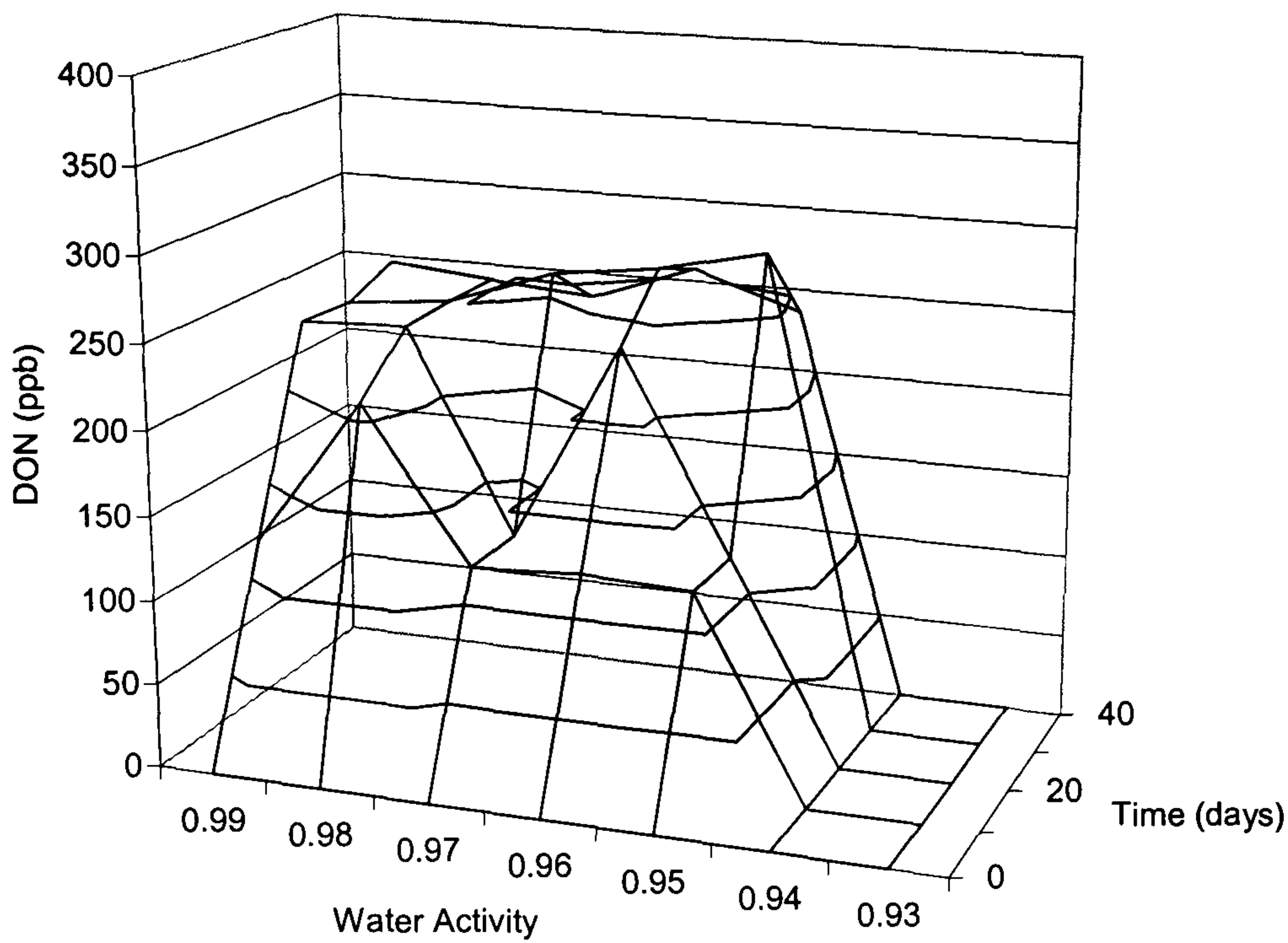


**Figure 3.3** *F. culmorum* growth on wheat agar adjusted to various  $a_w$  levels and incubated at ■ 25 °C and □ 15 °C.

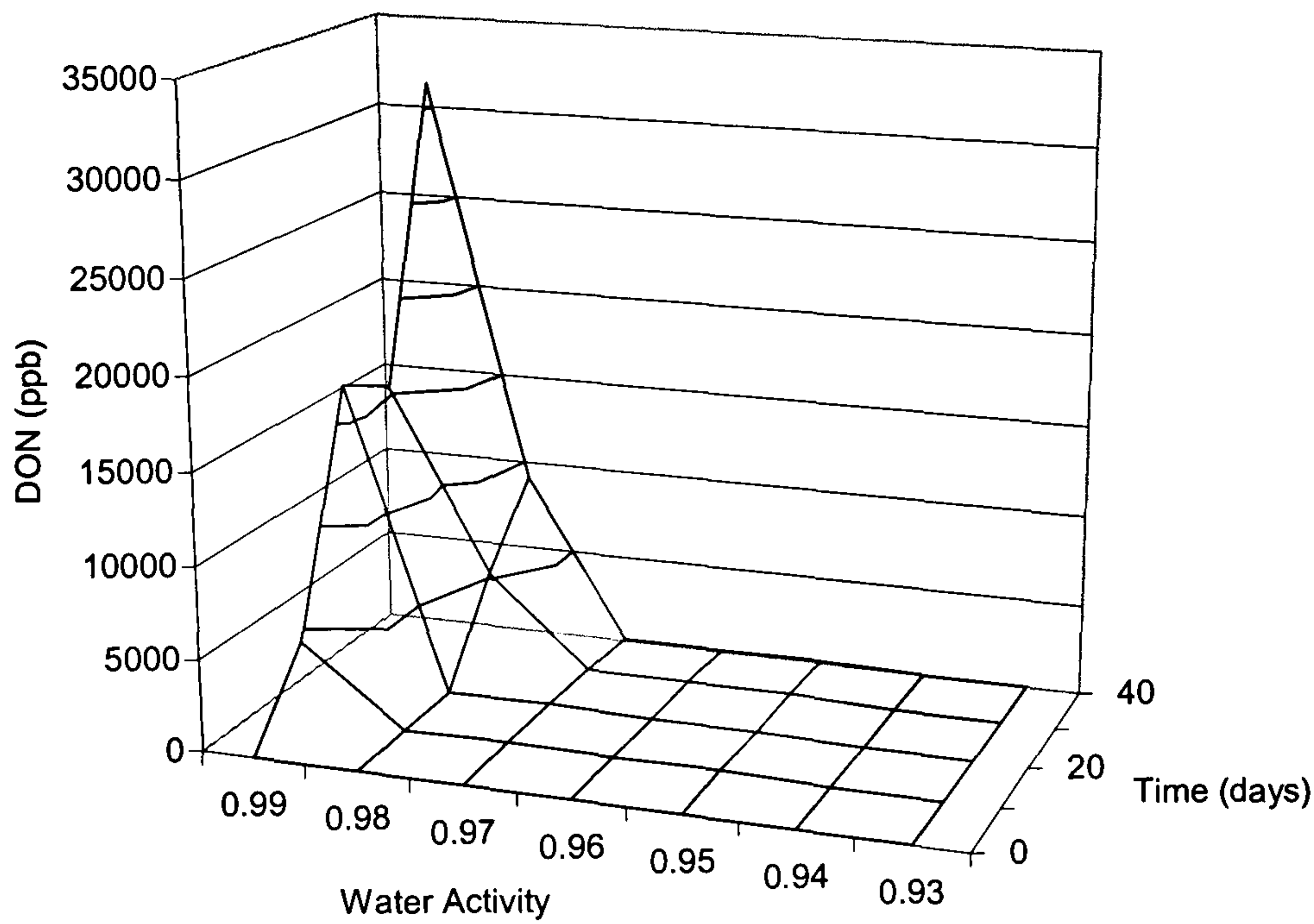
**Table 3.1** Significance test of experimental factors’ effect on *F. culmorum* growth. \* Indicates factor elicited a significant effect (P <0.05).

Source of Variation	DF	SS	MS	F	P
Temp	1	1.452	1.452	57.834	<0.001*
$a_w$	6	292.548	48.758	1942.128	<0.001*
Temp x $a_w$	6	8.467	1.411	56.213	<0.001*
Residual	28	0.703	0.0251		
Total	41	303.171	7.394		



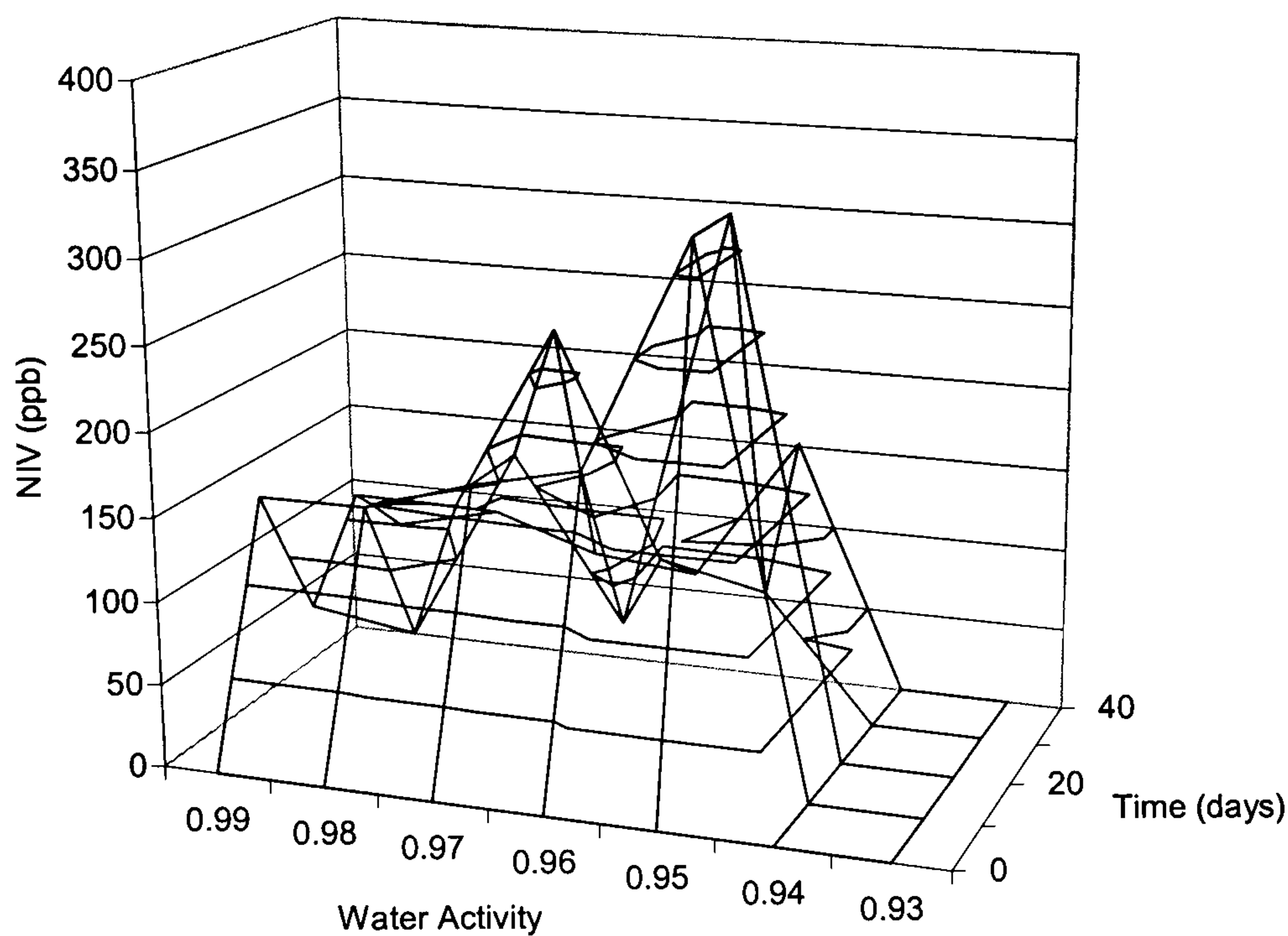


**Figure 3.4** *F. culmorum* production of DON on wheat agar at various  $a_w$  levels, incubated at 15 °C.

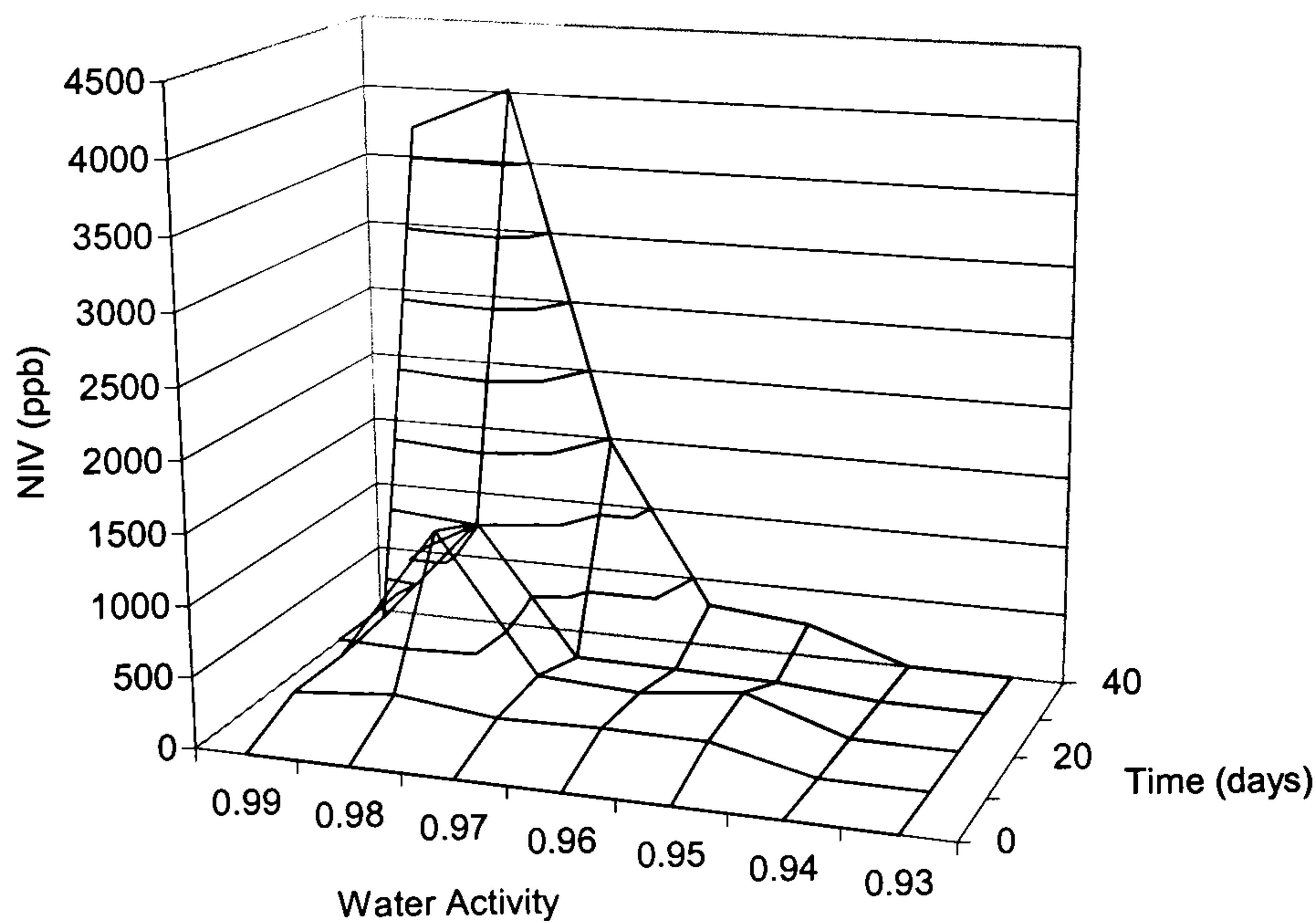


**Figure 3.5** *F. culmorum* production of DON on wheat agar at various  $a_w$  levels incubated at 25 °C.





**Figure 3.6** *F. culmorum* production of NIV on wheat agar at various  $a_w$  levels, incubated at 15 °C.



**Figure 3.7** *F. culmorum* production of NIV on wheat agar at various  $a_w$  levels, incubated at 25 °C.



**Table 3.2** Significance test of experimental factors’ effect on production of DON by *F. culmorum*. \* Indicates factor elicited a significant effect (P <0.05).

Source of Variation	DF	SS	MS	F	P
a <sub>w</sub>	6	9.14E+08	1.52E+08	22.547	<0.001*
Incubation temp	1	2.47E+08	2.47E+08	36.573	<0.001*
Incubation time	3	64160022	21386674	3.164	0.027*
a <sub>w</sub> x Incubation temp	6	9.07E+08	1.51E+08	22.358	<0.001*
a <sub>w</sub> x Incubation time	18	2.4E+08	13318528	1.971	0.017*
Incubation temp x Incubation time	3	62325982	20775327	3.074	0.031*
a <sub>w</sub> x Incubation temp x Incubation time	18	2.44E+08	13561221	2.007	0.015*
Residual	112	7.57E+08	6758320		
Total	167	3.44E+09	20570943		

**Table 3.3.** Significance test of experimental factors’ effect on production of NIV by *F. culmorum*. \* Indicates factor elicited a significant effect (P <0.05).

Source of Variation	DF	SS	MS	F	P
a <sub>w</sub>	6	4434275	739045.8	2.265	0.042*
Incubation temp	1	4039240	4039240	12.381	<0.001*
Incubation time	3	2951526	983842.1	3.016	0.033*
a <sub>w</sub> x Incubation temp	6	5626451	937741.9	2.874	0.012*
a <sub>w</sub> x Incubation time	18	5302771	294598.4	0.903	0.576
Incubation temp x Incubation time	3	4682219	1560740	4.784	0.004*
a <sub>w</sub> x Incubation temp x Incubation time	18	5010894	278383	0.853	0.635
Residual	112	36539414	326244.8		
Total	167	68586790	410699.3		

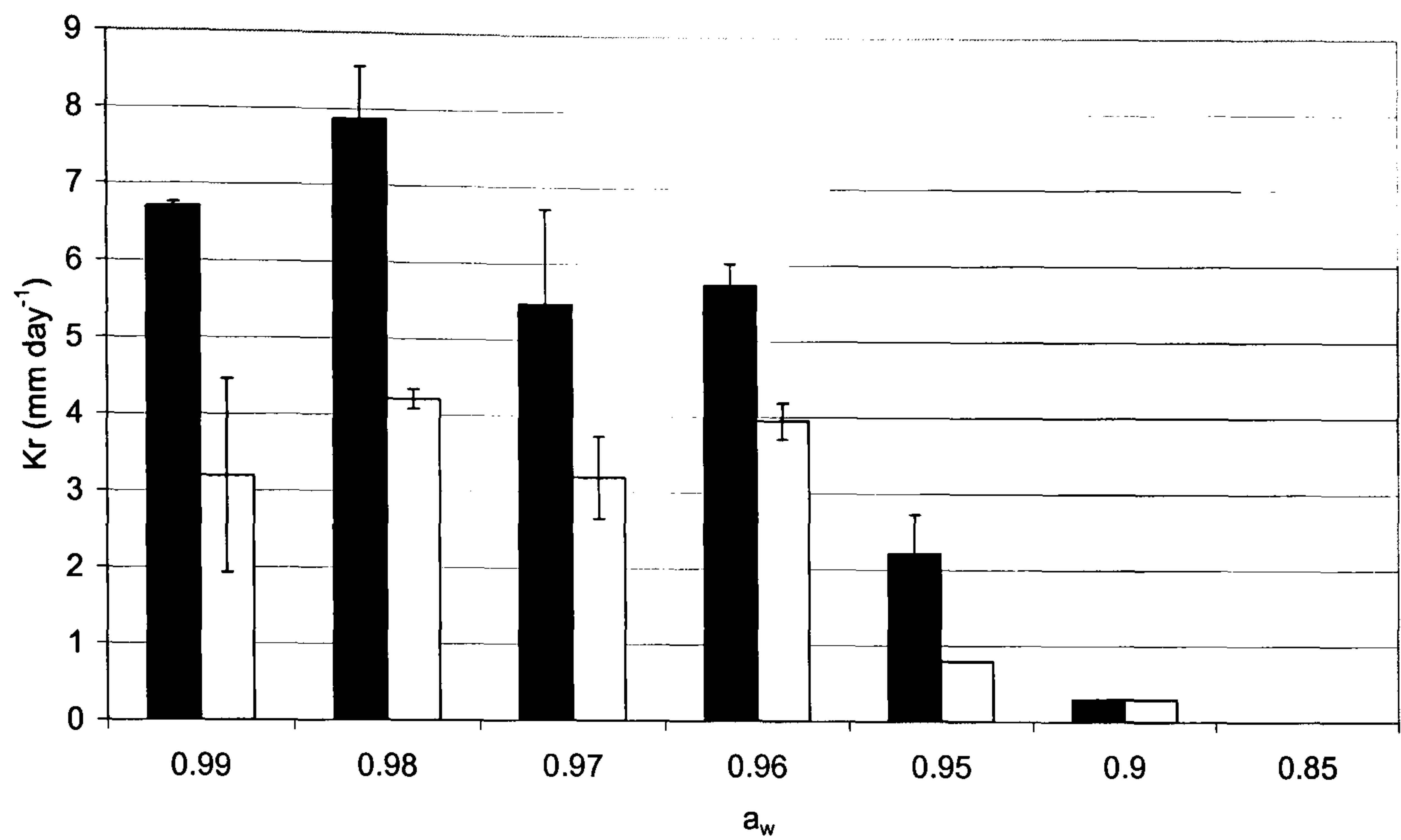


### 3.2.2 *F. culmorum* growth and DON/NIV production on irradiated wheat grain

Growth rates obtained for *F. culmorum* on irradiated wheat grain are shown in Figure 3.8. The highest growth rates were obtained at 0.98a<sub>w</sub> and 25°C. The effects on growth at a<sub>w</sub> levels between 0.99 - 0.96 a<sub>w</sub> were smaller than those observed on agar in the same a<sub>w</sub> range. Variation between replicates was also larger than those observed on agar substrate. ANOVA (Table 3.4) proved that all the experimental factors and their interactions had a significant effect on growth.

Figure 3.9 and 3.10 display the production of DON by *F. culmorum* on irradiated wheat grain at 15 °C and 25 °C respectively. At 25 °C the highest levels of DON were produced at 0.99 a<sub>w</sub> after 40 days. The highest DON levels achieved at 15 °C was at 0.97 a<sub>w</sub> after 40 days. NIV was detected in many of the samples; however, it was only present at levels lower than the level of quantification. ANOVA (Table 3.5) of the DON results show that all the factors and their interactions were significant.



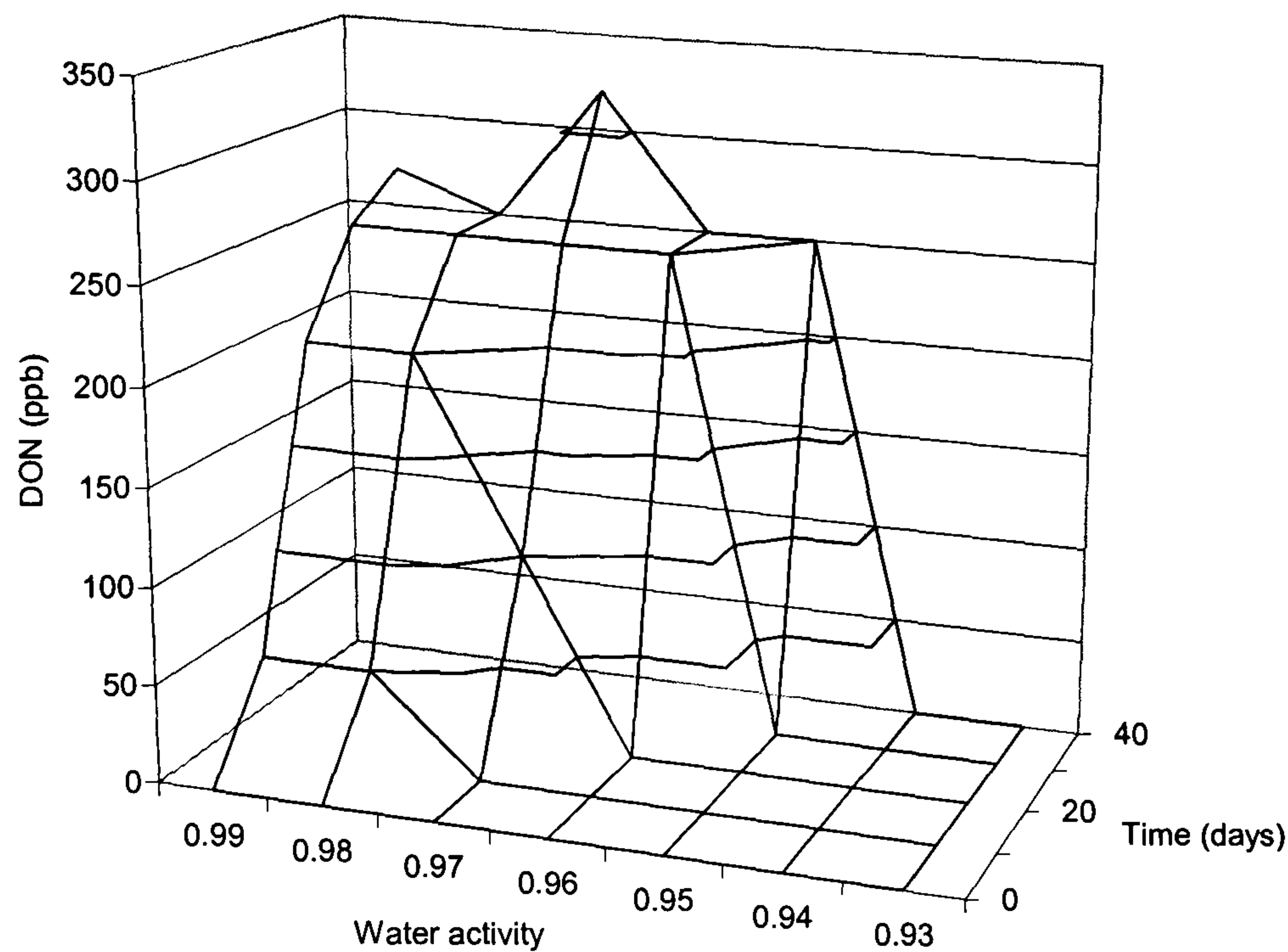


**Figure 3.8** *F. culmorum* growth on irradiated wheat grain at various a<sub>w</sub> levels and incubated at ■ 25 °C and □ 15 °C

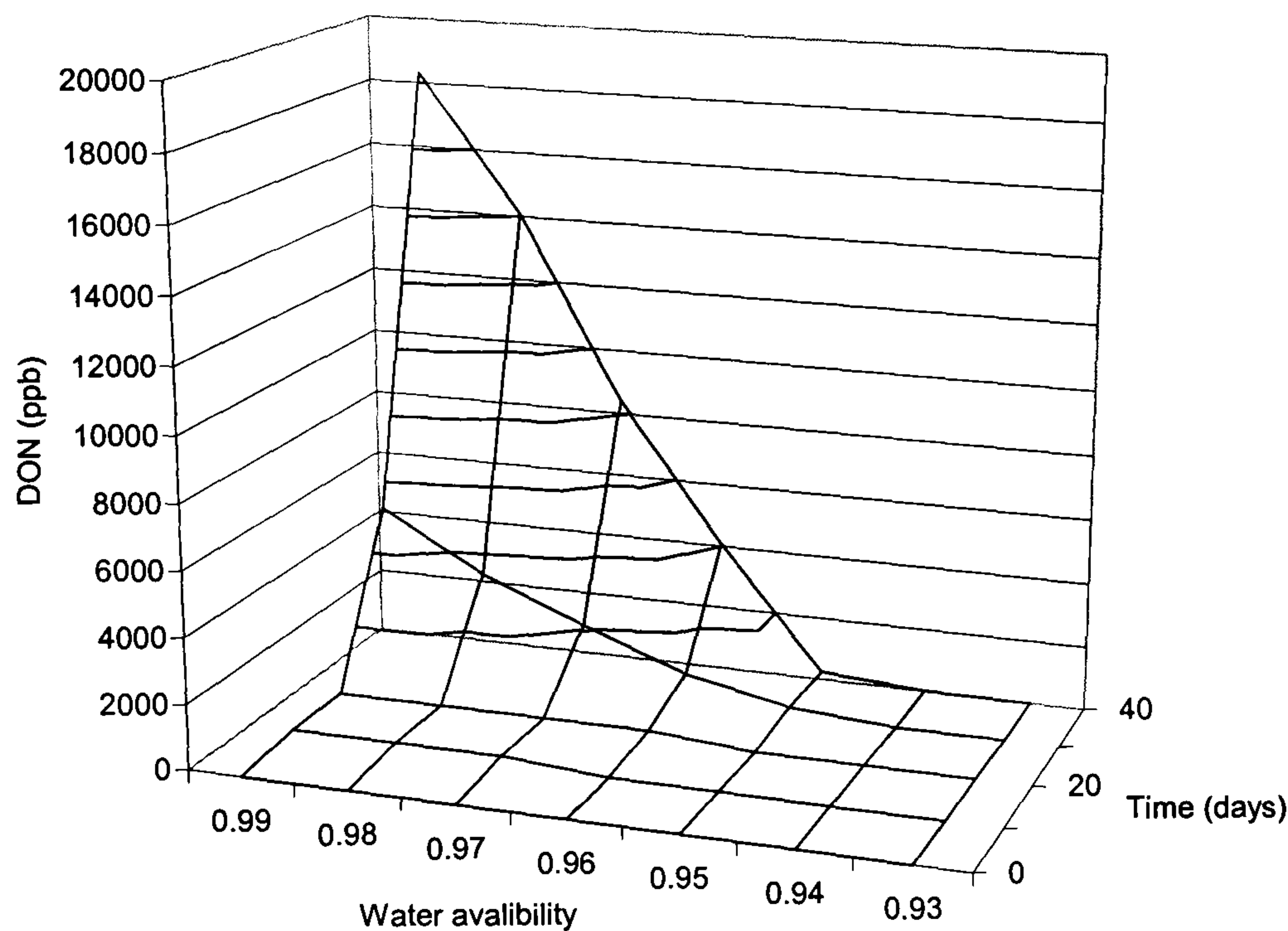
**Table 3.4** ANOVA for *F. culmorum* growth on irradiated wheat grain. \* Indicates factor elicited a significant effect (P <0.05).

Source of Variation	DF	SS	MS	F	P
Temp	1	36.905	36.905	119.34	<0.001*
a <sub>w</sub>	6	232.544	38.757	125.33	<0.001*
Temp x a <sub>w</sub>	6	22.335	3.722	12.04	<0.001*
Residual	28	8.659	0.309		
Total	41	300.442			





**Figure 3.9** *F. culmorum* DON production on irradiated wheat grain at various  $a_w$  levels, and 15 °C.



**Figure 3.10** *F. culmorum* DON production on irradiated wheat grain at various  $a_w$  levels and at 25 °C.



**Table 3.5** ANOVA for *F. culmorum* DON production on irradiated wheat grain. \*

Indicates factor elicited a significant effect (P <0.05).

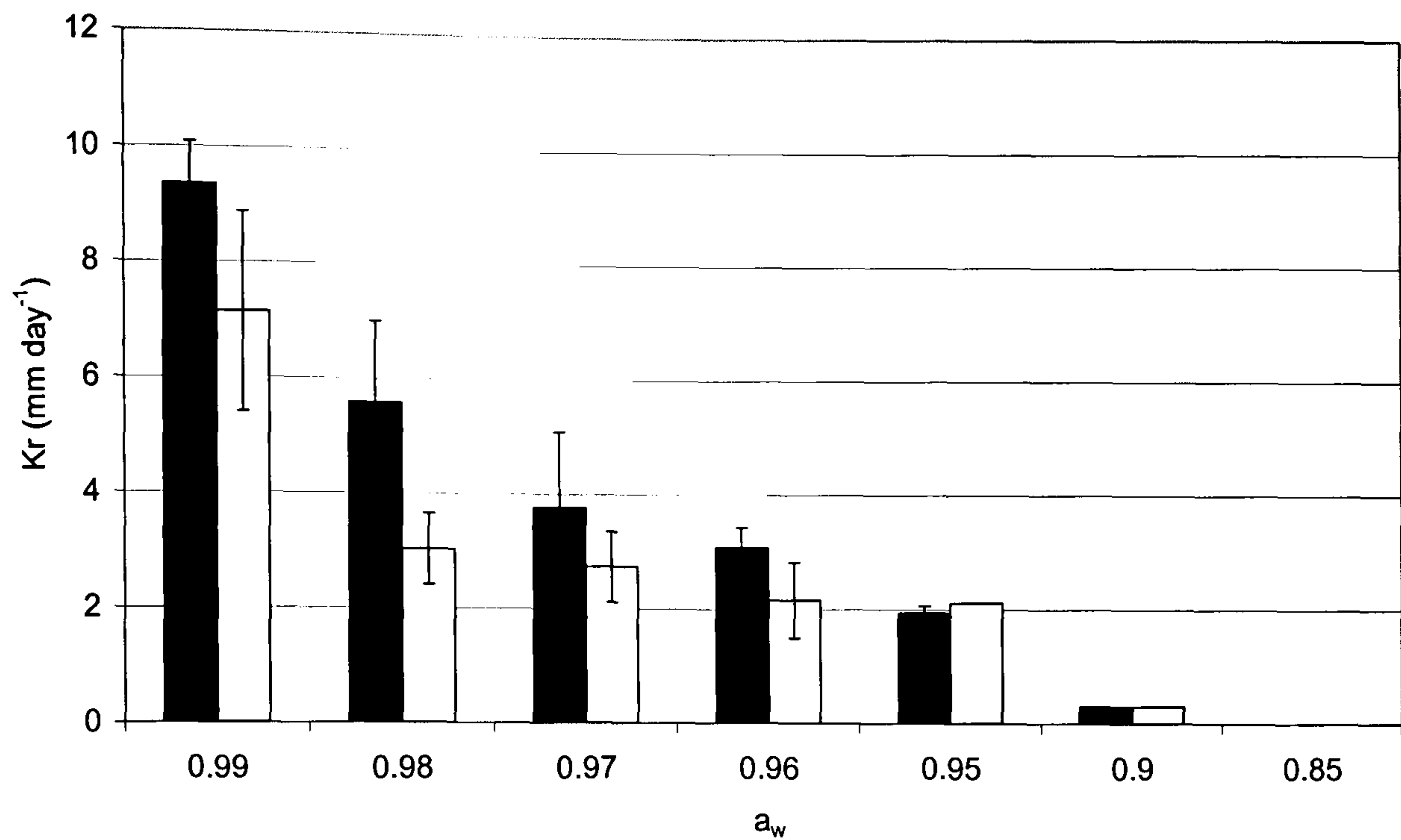
Source of Variation	DF	SS	MS	F	P
a <sub>w</sub>	6	2.21E+08	36865918	1240.1	<0.001*
Temp	1	1.67E+08	1.67E+08	5616.93	<0.001*
Time	3	2.95E+08	98399720	3309.97	<0.001*
a <sub>w</sub> x Temp	6	1.96E+08	32705411	1100.15	<0.001*
a <sub>w</sub> x Time	18	3.25E+08	18052382	607.25	<0.001*
Temp x Time	3	2.69E+08	89707405	3017.58	<0.001*
a <sub>w</sub> x Temp x Time	18	3.21E+08	17817685	599.35	<0.001*
Residual	112	3329564	29728		
Total	167	1.8E+09			



### 3.2.3 Effect of temperature x $a_w$ interactions, on growth and DON/NIV production by *F. graminearum* on wheat agar

Figure 3.11 shows the growth rates determined for *F. graminearum* on wheat agar. The highest growth rate was attained at 0.99  $a_w$  and 25 °C. No growth was observed below 0.9  $a_w$  regardless of temperature. Temperature significantly affected growth at  $a_w$  levels greater than 0.95  $a_w$ . Below this  $a_w$  level, temperature did not elicit a significant effect on growth. ANOVA (Table 3.6) showed that all the experimental factors and their interactions had a significant effect on growth.

Figures 3.12 and 3.13 show the temporal DON production profile for *F. graminearum* incubated at 15 and 25 °C. DON levels generally increased with  $a_w$ , temperature and time. The optimal conditions for DON production was 0.99  $a_w$  and 25 °C. At 15 °C DON production was highest at 0.98  $a_w$ . NIV production is displayed graphically in Figures 3.14 and 3.15. The highest levels of NIV were produced at 25 °C at 0.97  $a_w$  after 30 days incubation. At 15 °C the highest level of NIV was also produced at 0.97  $a_w$ . No mycotoxin was detected at under 0.95  $a_w$  at either temperature condition. ANOVA (Tables 3.7 and 3.8) shows that all the factors and their interactions had a significant effect on DON and NIV levels.

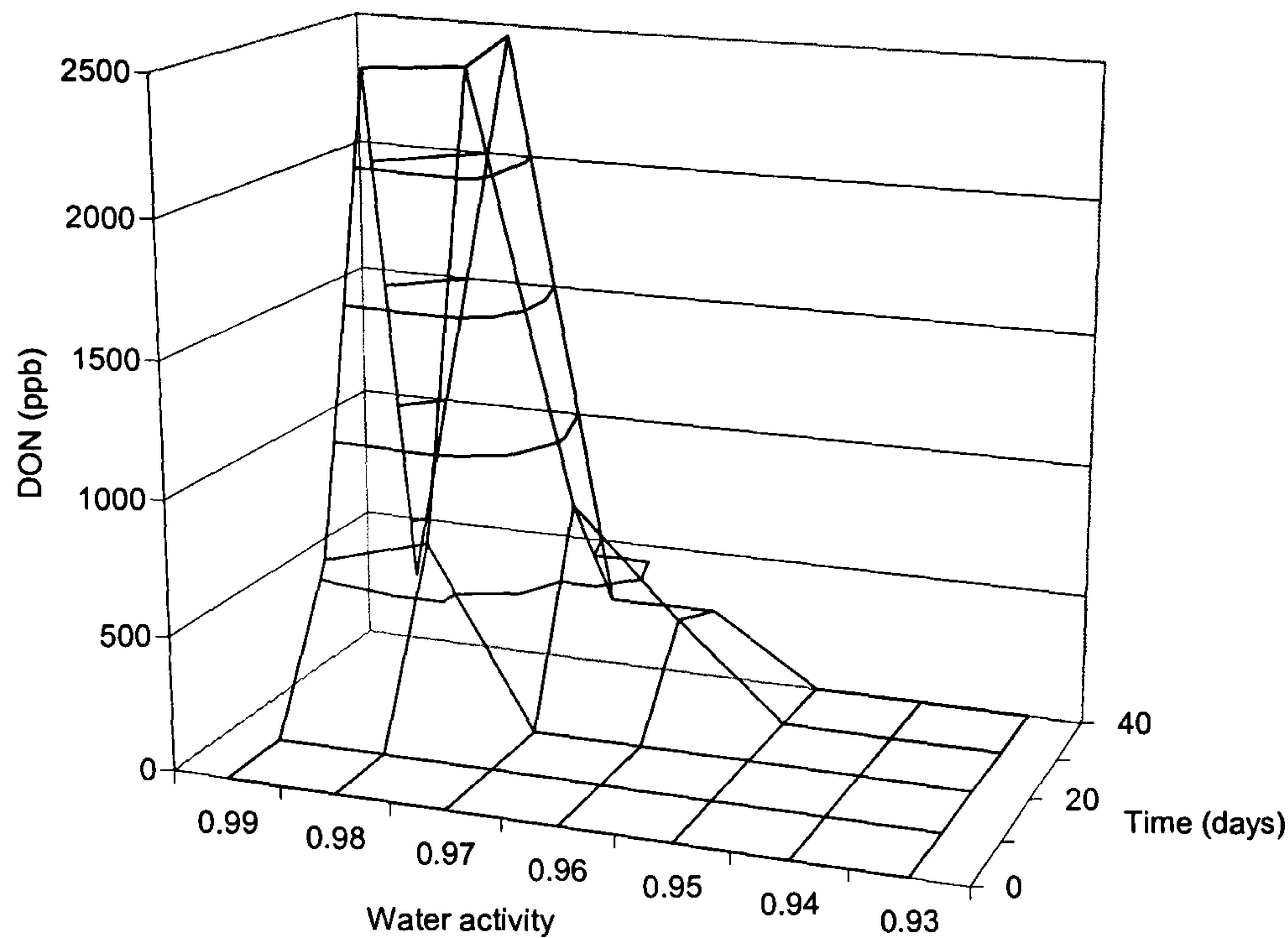


**Figure 3.11** *F. graminearum* growth on wheat agar at various  $a_w$  levels, incubated at ■ 25 °C and □ 15 °C.

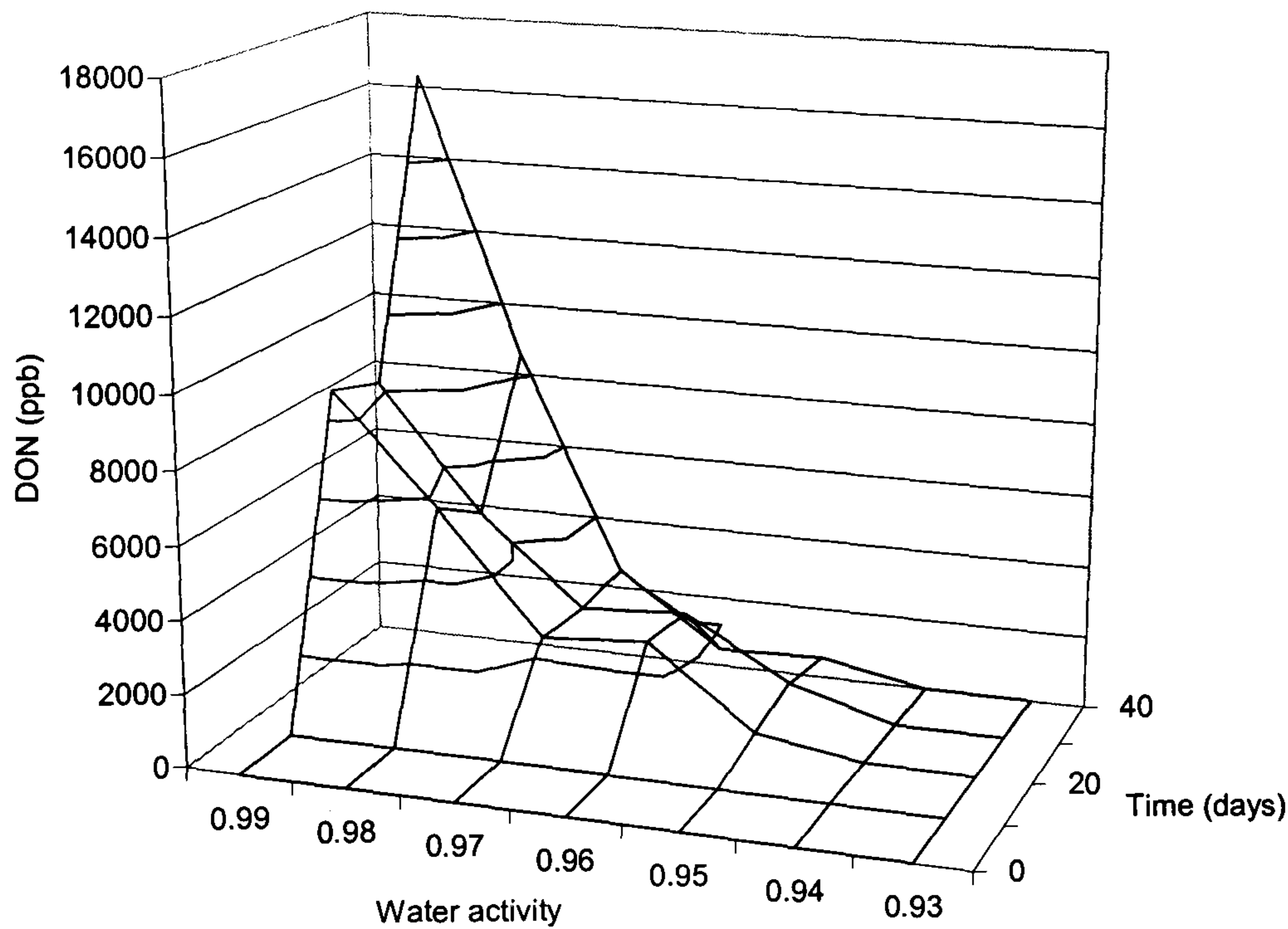
**Table 3.6** ANOVA of *F. graminearum* growth on wheat agar. \* Indicates factor elicited a significant effect (P <0.05).

Source of Variation	DF	SS	MS	F	P
$a_w$	6	236.093	39.349	63.45	<0.001*
temp	1	10.835	10.835	17.47	<0.001*
$a_w$ x temp	6	14.021	2.337	3.77	0.007*
Residual	28	17.364	0.62		
Total	41	278.313			

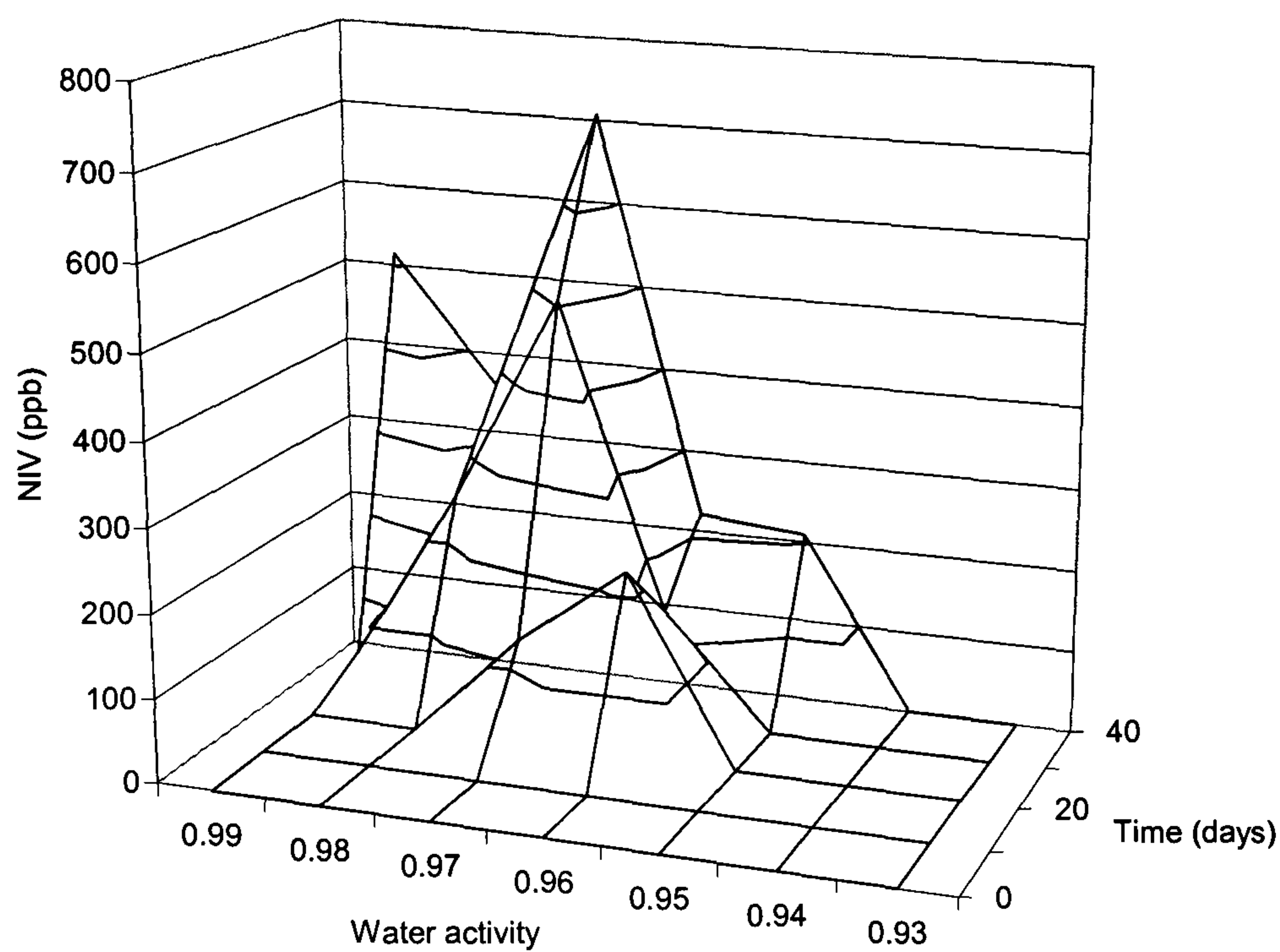




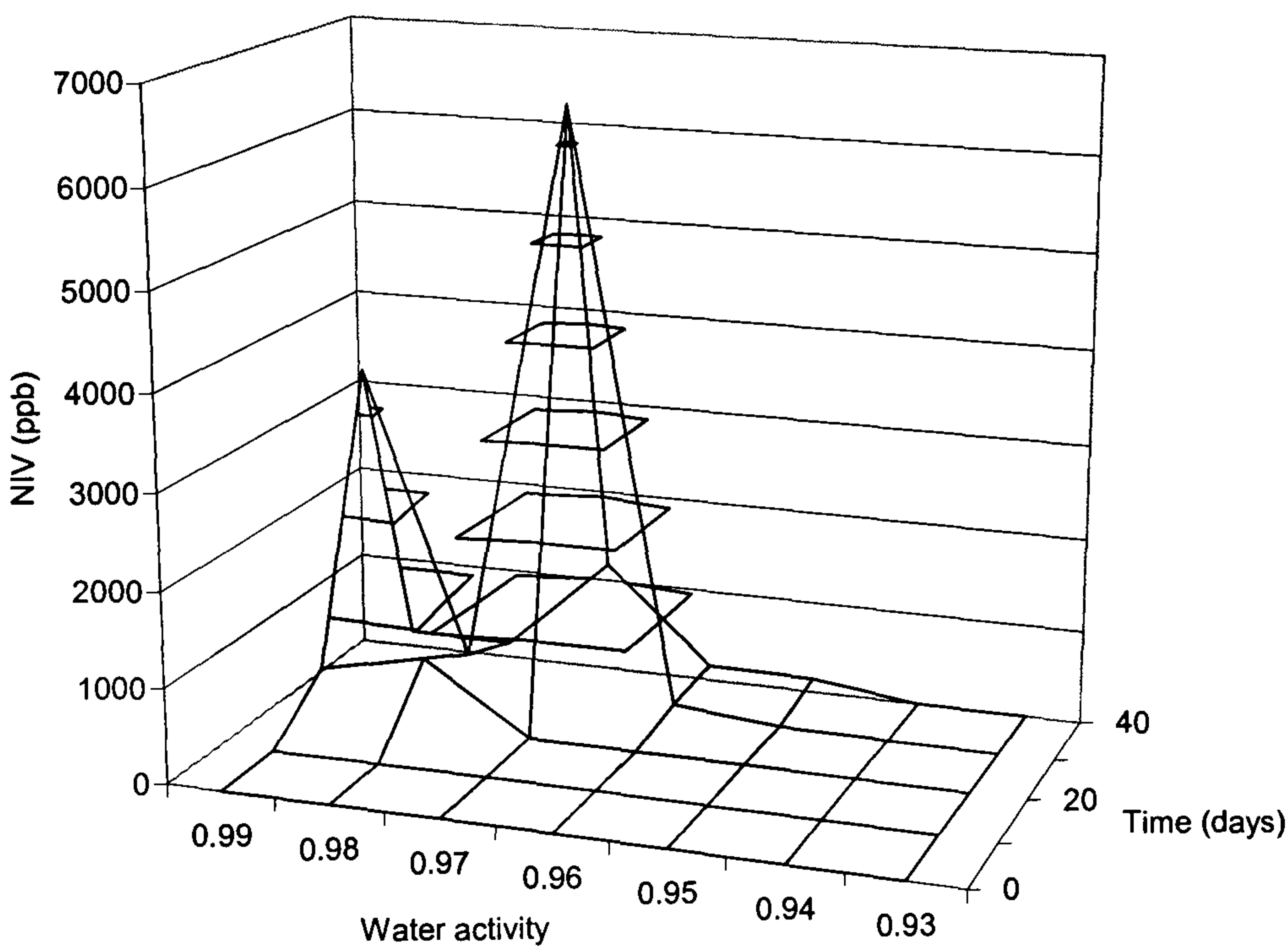
**Figure 3.12** *F. graminearum* DON production on wheat agar, at various  $a_w$  levels and 15 °C.



**Figure 3.13** *F. graminearum* DON production on wheat agar, at various  $a_w$  levels and 25 °C.



**Figure 3.14** NIV production by *F. graminearum* on wheat agar at various  $a_w$  levels and 15 °C.



**Figure 3.15** NIV production by *F. graminearum* on wheat agar, at various  $a_w$  levels and 25 °C.



**Table 3.7** ANOVA of DON production by *F. graminearum* on wheat agar. \*

Indicates factor elicited a significant effect (P <0.05).

Source of Variation	DF	SS	MS	F	P
a <sub>w</sub>	6	4.46E+08	74304404	2820.51	<0.001*
Incu temp	1	1.72E+08	1.72E+08	6534.91	<0.001*
Incu time	3	1.19E+08	39707900	1507.27	<0.001*
a <sub>w</sub> x Incu temp	6	2.61E+08	43475453	1650.28	<0.001*
a <sub>w</sub> x Incu time	18	2.04E+08	11350419	430.85	<0.001*
Incu temp x Incu time	3	74021022	24673674	936.59	<0.001*
a <sub>w</sub> x Incu temp x Incu time	18	1.73E+08	9608408	364.72	<0.001*
Residual	112	2950561	26344		
Total	167	1.45E+09			

**Table 3.8** ANOVA of NIV production by *F. graminearum* on wheat agar. \* Indicates factor elicited a significant effect (P <0.05).

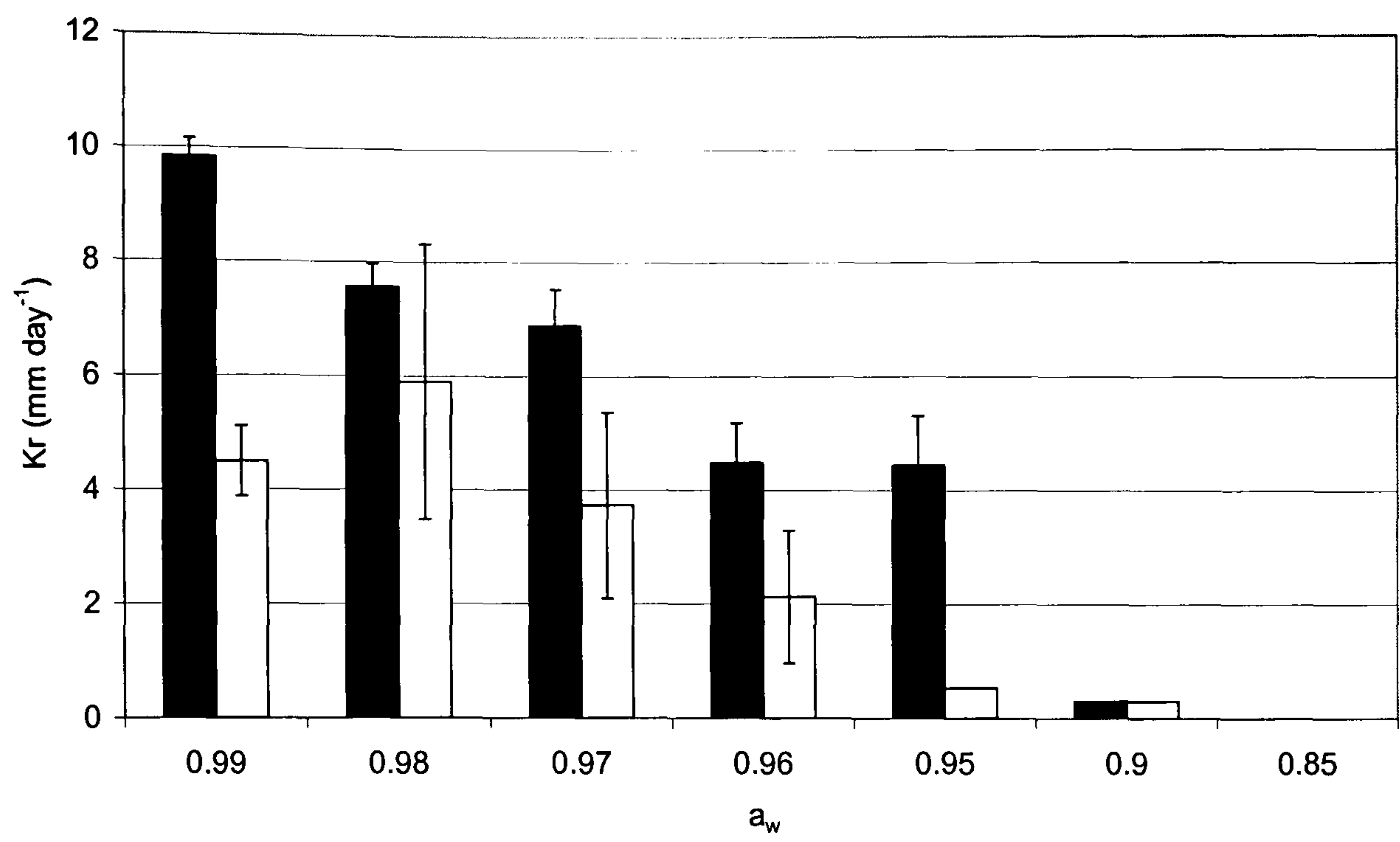
Source of Variation	DF	SS	MS	F	P
a <sub>w</sub>	6	24820725	4136787	1629.81	<0.001*
Incu temp	1	5490439	5490439	2163.12	<0.001*
Incu time	3	16077192	5359064	2111.36	<0.001*
a <sub>w</sub> x Incu temp	6	14152482	2358747	929.3	<0.001*
a <sub>w</sub> x Incu time	18	43039349	2391075	942.03	<0.001*
Incu temp x Incu time	3	13757546	4585849	1806.73	<0.001*
a <sub>w</sub> x Incu temp x Incu time	18	38258033	2125446	837.38	<0.001*
Residual	112	284279	2538		
Total	167	1.56E+08			

### 3.2.4 Effect of temperature x $a_w$ interactions, on growth and DON/NIV production by *F. graminearum* on irradiated wheat grain

Growth rates obtained for *F. graminearum* on irradiated wheat grain are shown in Figure 3.16. The highest growth rates were obtained at 0.99  $a_w$  and 25 °C. No growth was observed at  $a_w$  levels below 0.90  $a_w$ . The effects on growth at  $a_w$  levels between 0.99 - 0.97  $a_w$  was smaller than those observed on agar in the same  $a_w$  range. Variation between replicates was also larger than those observed on agar substrate at both 25 °C and 15 °C. ANOVA (Table 3.9) showed that all the experimental factors and their interactions had a significant effect on growth.

Figures 3.17 and 3.18 display the production of DON by *F. graminearum* on irradiated wheat grain at 25 °C and 15 °C respectively. The highest levels of DON were produced at 0.99  $a_w$  after 40 days at both 25 °C and 15 °C. NIV was detected in many of the samples; however, it was again only present at levels lower than the level of quantification. ANOVA (Table 3.10) of the DON results show that all the factors and their interactions were significant.

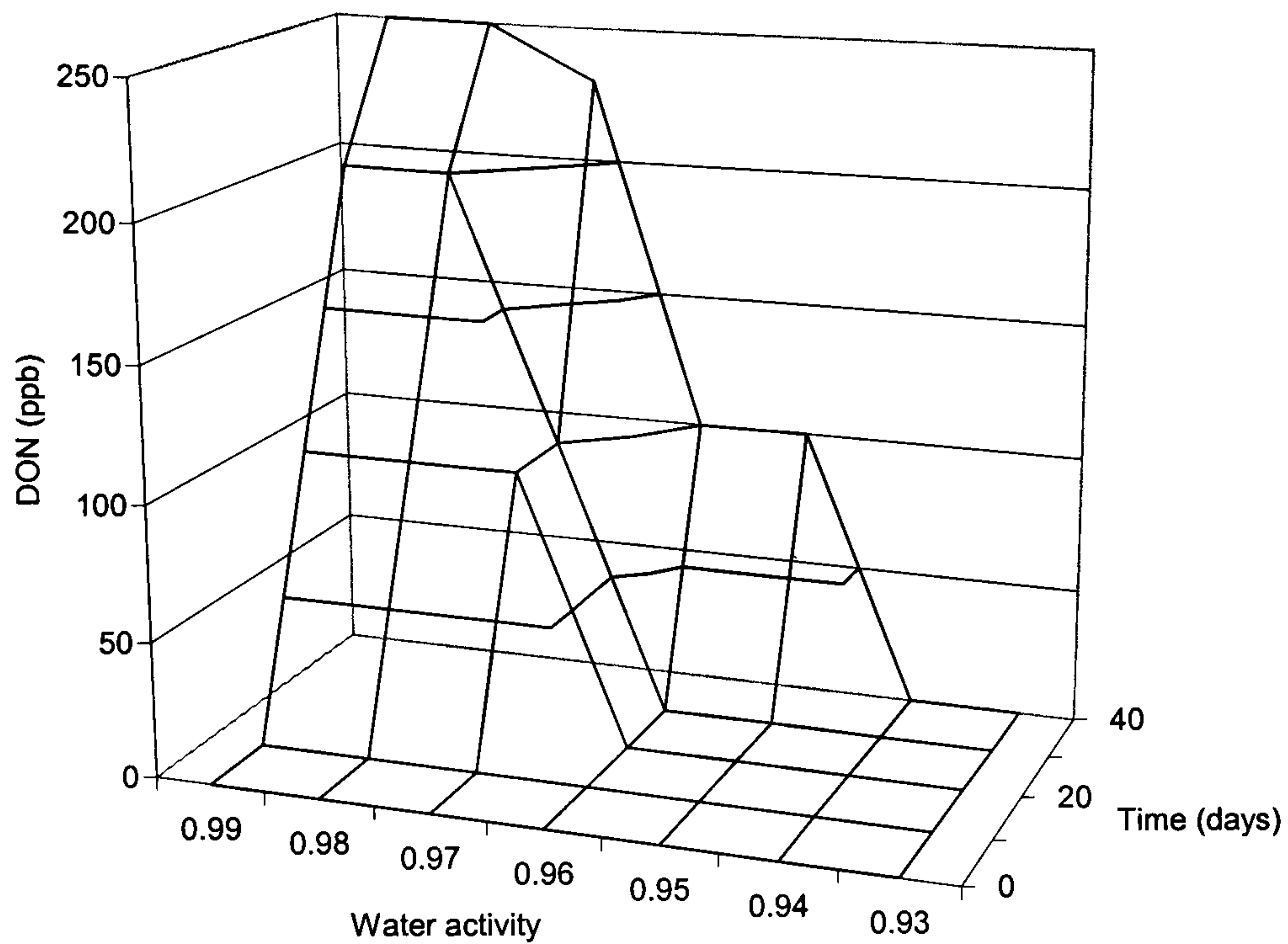




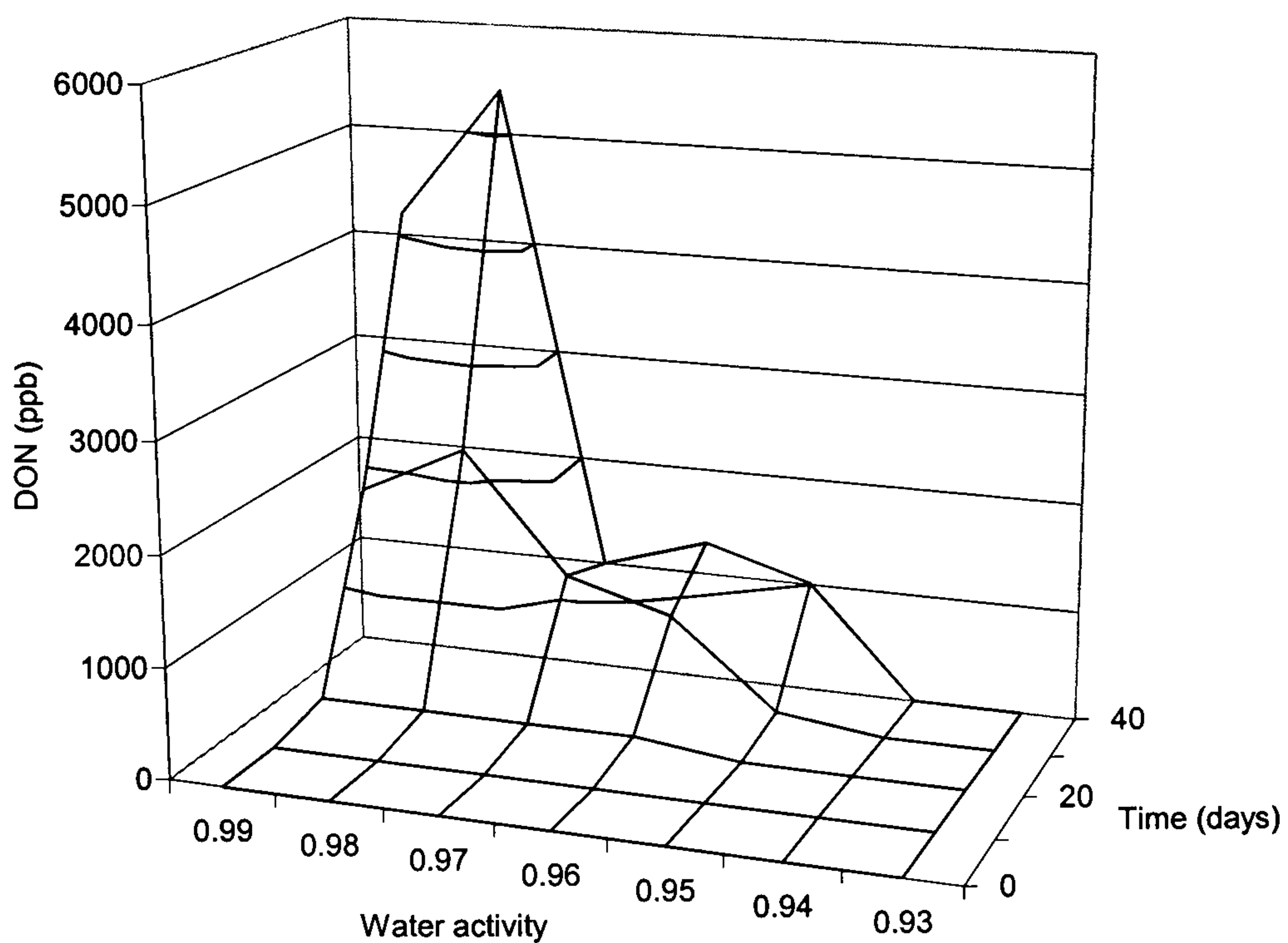
**Figure 3.16** *F. graminearum* growth rates at various  $a_w$  levels and two different temperatures (■ 25 °C □ 15 °C) on an irradiated wheat grain substrate.

**Table 3.9** ANOVA for *F. graminearum* growth on irradiated wheat grain.

Source of Variation	DF	SS	MS	F	P
Temp	1	77.727	77.727	89.37	<0.001
$a_w$	6	270.898	45.15	51.91	<0.001
Temp x $a_w$	6	42.22	7.037	8.09	<0.001
Residual	28	24.352	0.87		
Total	41	415.197			



**Figure 3.17** DON production by *F. graminearum* on irradiated wheat grain at various  $a_w$  levels, at 15 °C.



**Figure 3.18** DON production by *F. graminearum* on irradiated wheat grain at various  $a_w$  levels, at 25 °C.



**Table 3.10** ANOVA for *F. graminearum* DON production on irradiated wheat grain

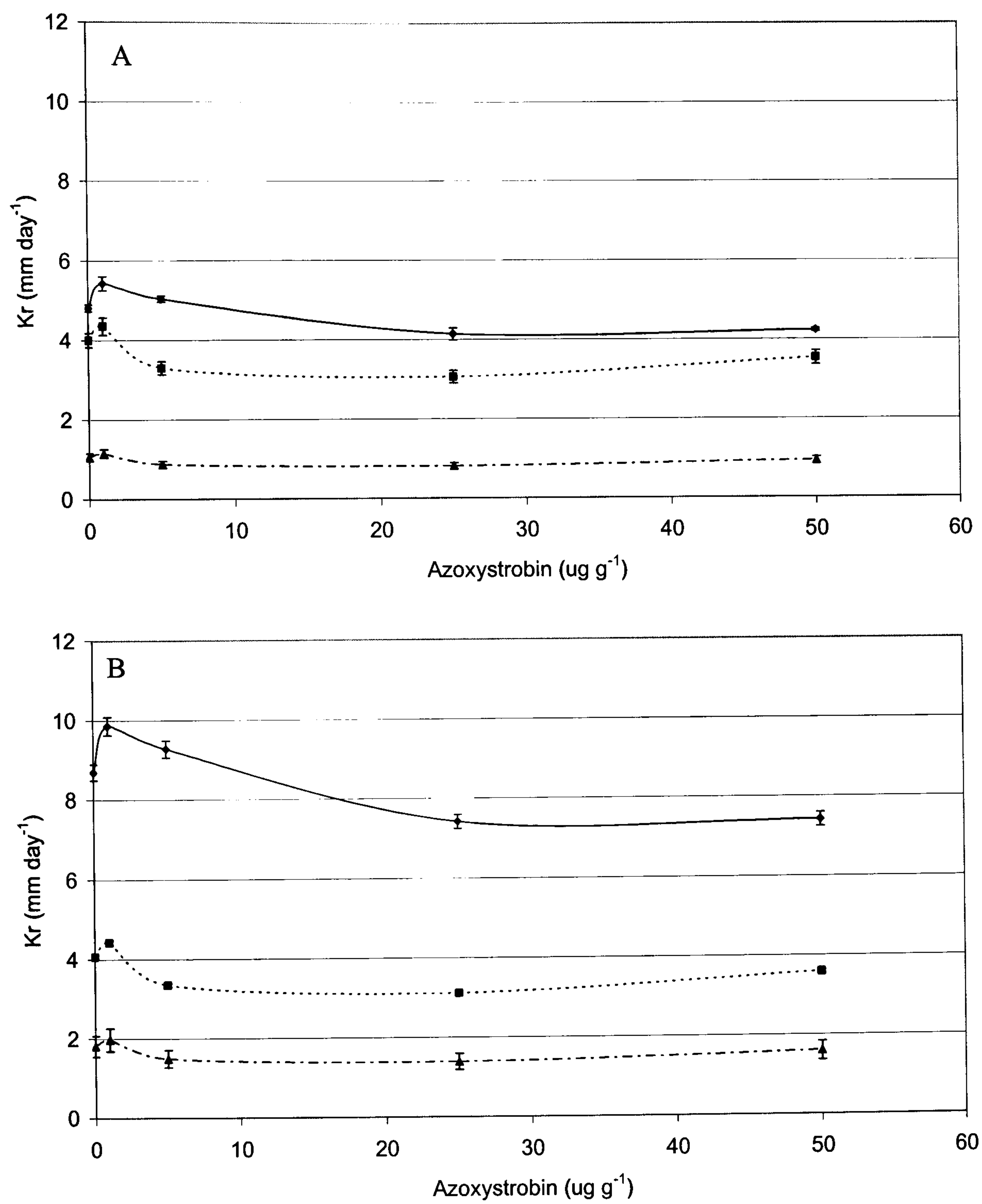
Source of Variation	DF	SS	MS	F	P
a <sub>w</sub>	6	23799665	3966611	1444.86	<0.001
Temp	1	17197440	17197440	6264.26	<0.001
Time	3	26509447	8836482	3218.73	<0.001
a <sub>w</sub> x Temp	6	17613823	2935637	1069.32	<0.001
a <sub>w</sub> x Time	18	28937348	1607630	585.59	<0.001
Temp x Time	3	20694699	6898233	2512.72	<0.001
a <sub>w</sub> x Temp x Time	18	25252760	1402931	511.02	<0.001
Residual	112	307477	2745		
Total	167	1.6E+08			

### 3.3 EFFECT OF COMMON FUNGICIDES ON *F. CULMORUM* GROWTH

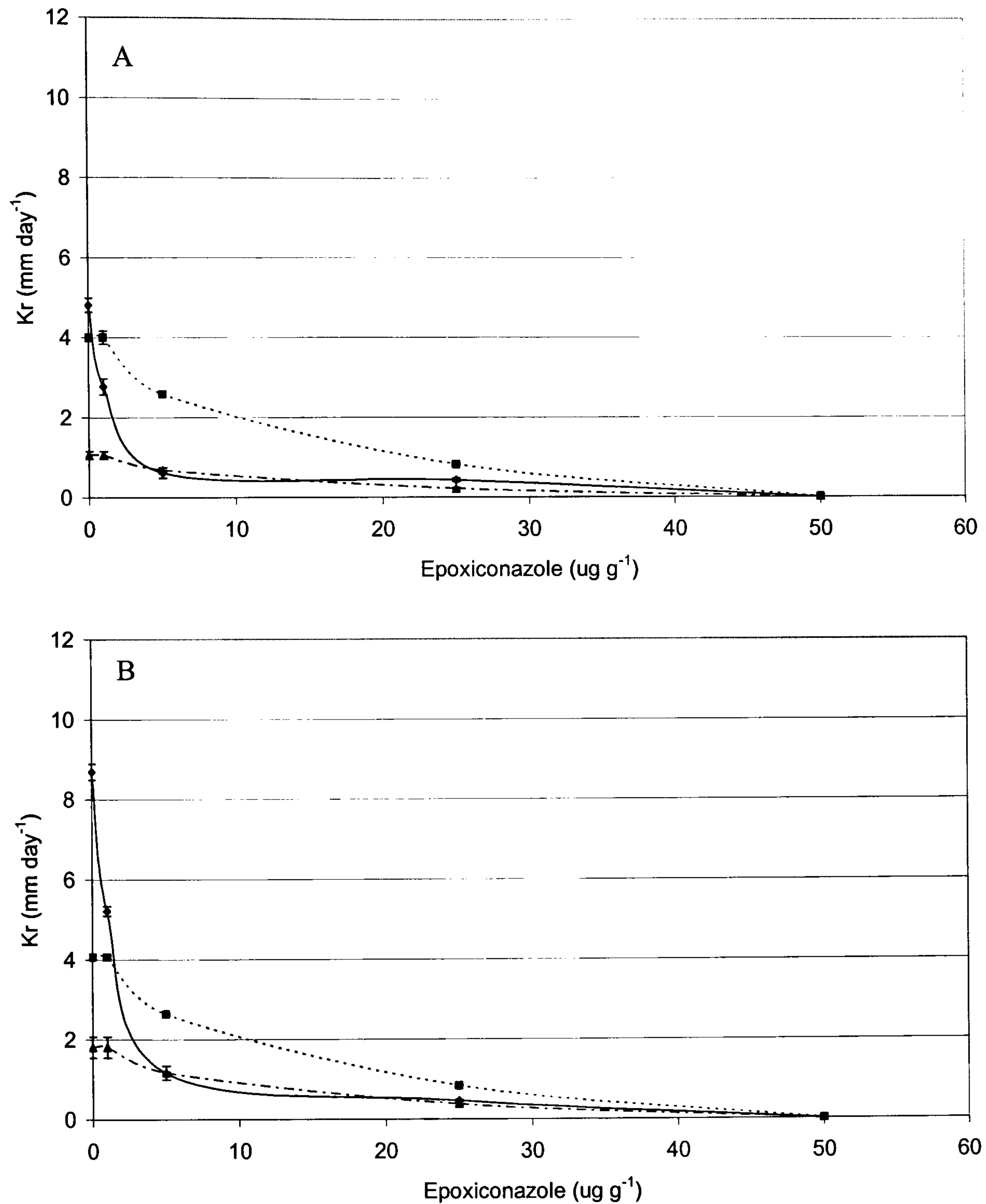
The literature review discussed how fungal spoilage in grain is currently controlled with fungicides and effective drying regimes in storage. Previous experiments (sections 3.1 and 3.2) in this study have addressed the effects of temperature and  $a_w$  ranges on growth of *F. culmorum*, similar to those experienced during the drying and storage of wheat grain. This experiment investigated the effect that the presence of three common fungicides had on colonisation potential by *F. culmorum* under different environmental conditions.

Figure 3.19 shows the efficacy of azoxystrobin concentrations on controlling growth of *F. culmorum* on 2% wheat agar at various  $a_w$  levels and two temperatures. Azoxystrobin was unable to effectively control (<50 %) *F. culmorum* mycelial exstention, although there was a slight reduction in relative growth rates. Optimum reduction in growth occurred at  $25 \mu\text{g g}^{-1}$ ; increasing the concentration to  $50 \mu\text{g g}^{-1}$  resulted in either no significant change in growth rate or a slight increase. Stimulation of growth occurred in all conditions with azoxystrobin concentrations of  $1 \mu\text{g g}^{-1}$ . Figures 3.20 and 3.21 show the efficacies of propioconazole and epoxiconazole at controlling the growth of *F. culmorum*. Both propioconazole and epoxiconazole were able to cause large reductions in *F. culmorum* growth. Growth rate was generally reduced as fungicide concentration increased. The exception to this was at a concentration of  $1 \mu\text{g g}^{-1}$  where no significant reduction in growth was observed at 0.955  $a_w$ . Propioconazole was able to produce a >50 % reduction in growth at concentrations of  $10 \mu\text{g g}^{-1}$  or more in all of the  $a_w$  and temperature conditions. Epoxiconazole was also able to control growth by over 50 % at a concentration of  $2 \mu\text{g g}^{-1}$  but only at 0.995  $a_w$ . In order to achieve at least a 50 % reduction in *F. culmorum* growth with epoxiconazole when the  $a_w$  level was set to 0.98 and 0.955  $a_w$  concentrations of  $10 \mu\text{g g}^{-1}$  were required. *F. culmorum* growth was completely inhibited by  $50 \mu\text{g g}^{-1}$  of epoxiconazole under all conditions. Propioconazole completely inhibited growth at  $50 \mu\text{g g}^{-1}$  but only at 0.955  $a_w$  and 15 °C. The LD<sub>50</sub> for the three fungicides under the various  $a_w$  levels and temperatures tested are summarised in Table 3.11.



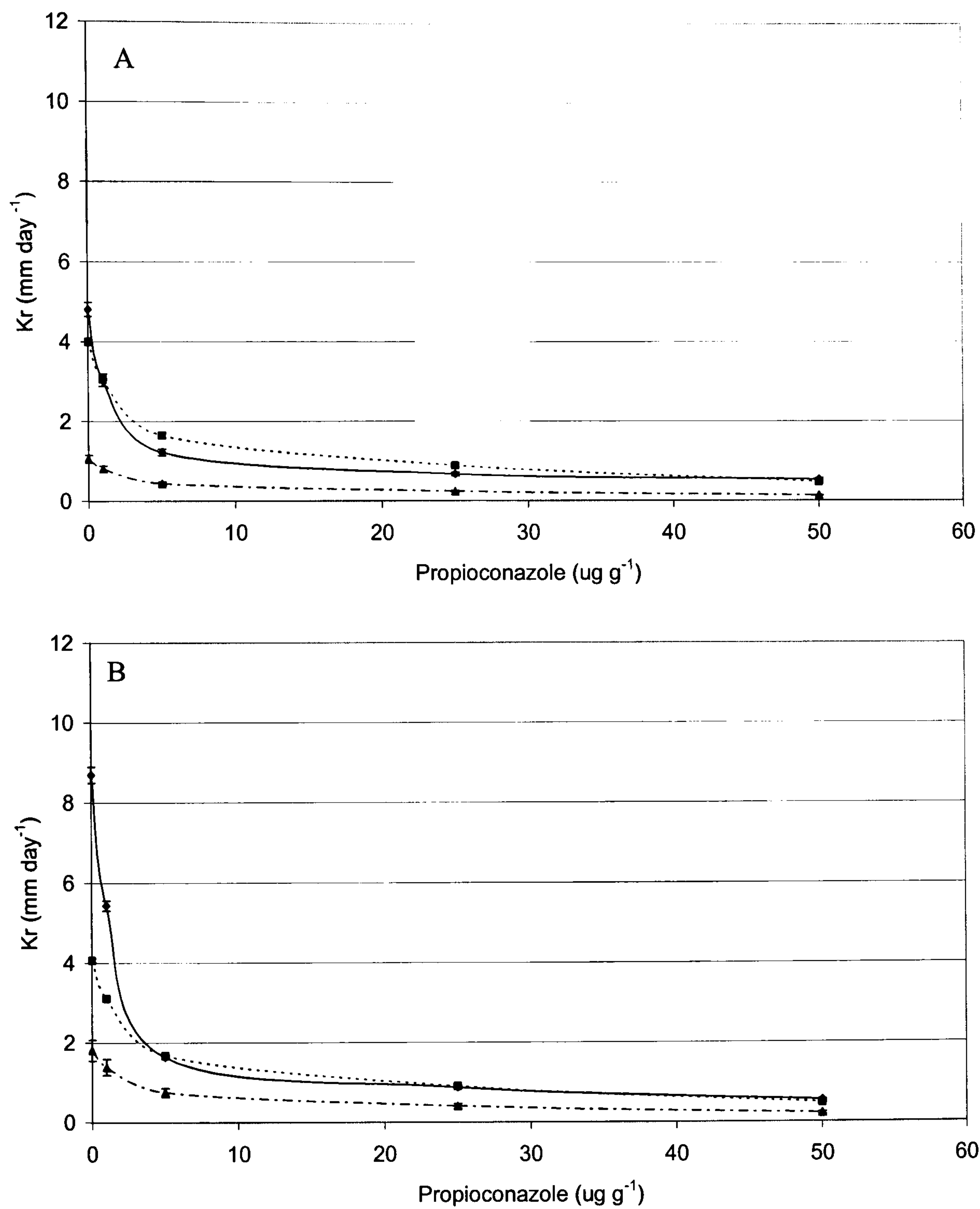


**Figure 3.19** *F. culmorum* growth on wheat agar supplemented with different concentration of azoxystrobin, incubated at 15 °C (A) and 25 °C (B) and adjusted to ◆ 0.995 a<sub>w</sub>, ■ 0.98 a<sub>w</sub>, ▲ 0.955 a<sub>w</sub>. Error bars represent standard error.



**Figure 3.20** *F. culmorum* growth on wheat agar supplemented with different concentration of epoxiconazole, incubated at 15 °C (A) and 25 °C (B) and adjusted to ◆ 0.995 a<sub>w</sub>, ■ 0.98 a<sub>w</sub>, ▲ 0.955 a<sub>w</sub>. Error bars represent standard error.





**Figure 3.21** *F. culmorum* growth on wheat agar supplemented with different concentration of propioconazole, incubated at 15 °C (A) and 25 °C (B) and adjusted to  $\blacklozenge$  0.995  $a_w$ ,  $\blacksquare$  0.98  $a_w$ ,  $\blacktriangle$  0.955  $a_w$ . Error bars represent standard error.

**Table 3.11** LD<sub>50</sub> levels for *F. culmorum* growth inhibition by three common wheat grain fungicides on wheat agar.

	15°C			25°C		
	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>
Azoxystrobin	N/A	N/A	N/A	N/A	N/A	N/A
Propioconazole	1.5	10	9.5	1.8	10.1	10
Epoxiconazole	2	3.5	7	1.9	3.4	3.4



### 3.4 INTERACTIONS BETWEEN *F. CULMORUM* AND OTHER SPOILAGE FUNGI ON WHEAT GRAIN BASED SUBSTRATES

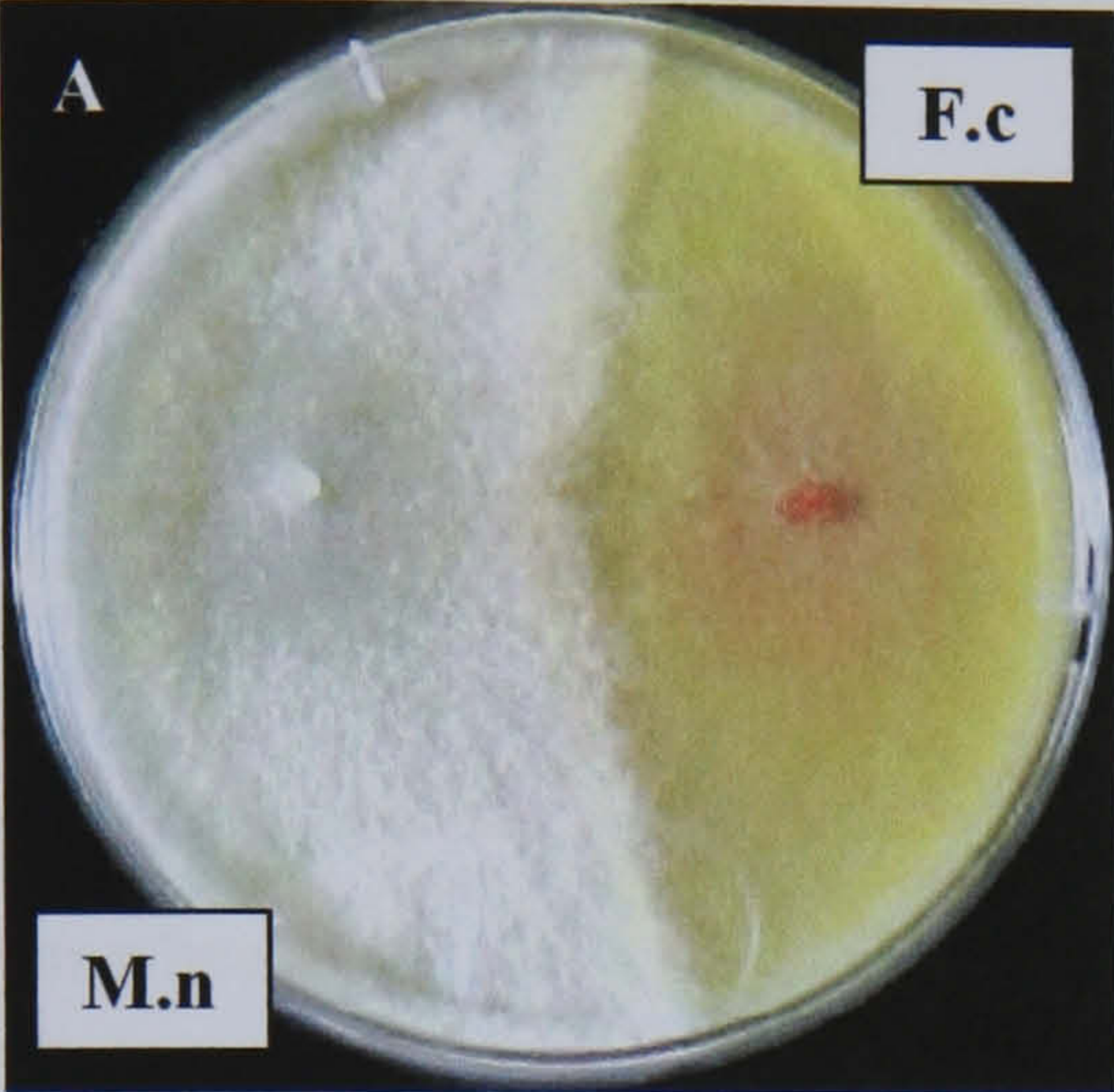
Interactions between different fungi have been shown to have a significant impact on growth, toxin production and population structure on cereal grain (Magan & Lacey, 1984a; Marin *et al.*, 1998c; Edwards *et al.*, 2001). The aims of this study were to:-

1. Determine the competitiveness of *F. culmorum* against a range of other wheat grain fungi on wheat agar and irradiated wheat grain substrates under various temperatures and water activity levels.
2. Investigate the effects of interspecific interactions under different environmental stresses on DON and NIV production by *F. culmorum*.

#### 3.4.1 Examples of macroscopic interaction

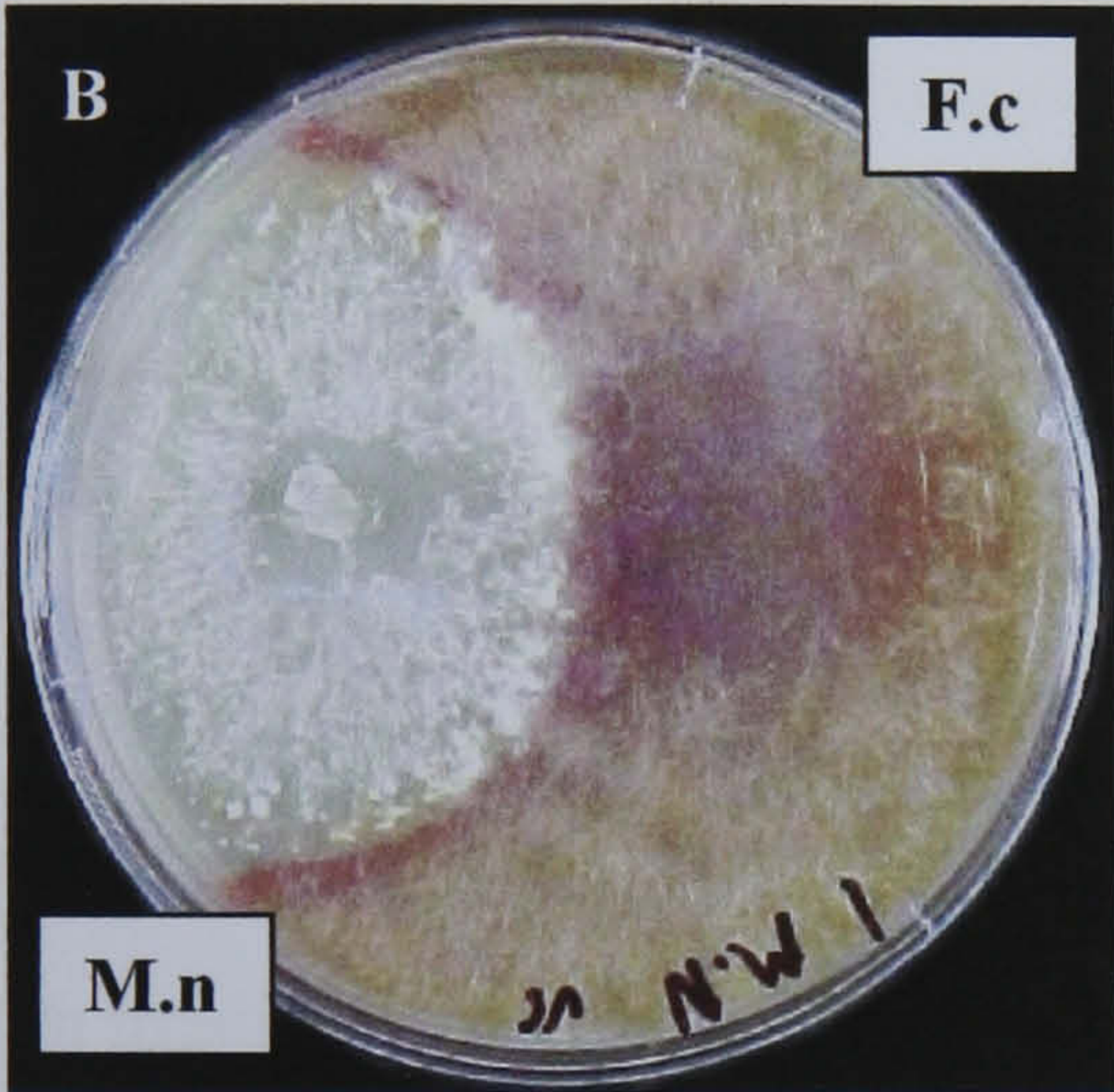
Plate 3.1, shows examples of dual culture plates used to determine macroscopic interactions between two species. The two species shown are *M. nivale* and *F. culmorum*. As the  $a_w$ , temperature and fungicide treatment were changed, the way in which the two species interact with each other also changed. When both these species were grown together at 0.98  $a_w$  and 15 °C on wheat agar their mycelium was able to intermingle. This is an example of mutual intermingling which is reflected in the interaction score by both fungi being given a score of 1. At 0.995  $a_w$  and 25 °C, *F. culmorum* and *M. nivale* aggressively defend their resources by preventing progression of each other at their colony perimeter. This is an example of inhibition on contact which is reflected in the interaction score for both fungi of 2. The addition of 0.5  $\mu\text{g g}^{-1}$  propiconazole to the medium in the last example allows *F. culmorum* to overcome *M. nivale*'s defences and continue growing into the region occupied by *M. nivale*. This is an example of dominance on contact which is reflected in the interaction score by *F. culmorum* being awarded 4 and *M. nivale* 0. A noteworthy change in the colonies of *F. culmorum* (UK3) is the production of dark pigmentation in the colony margin, adjacent to the competing species.





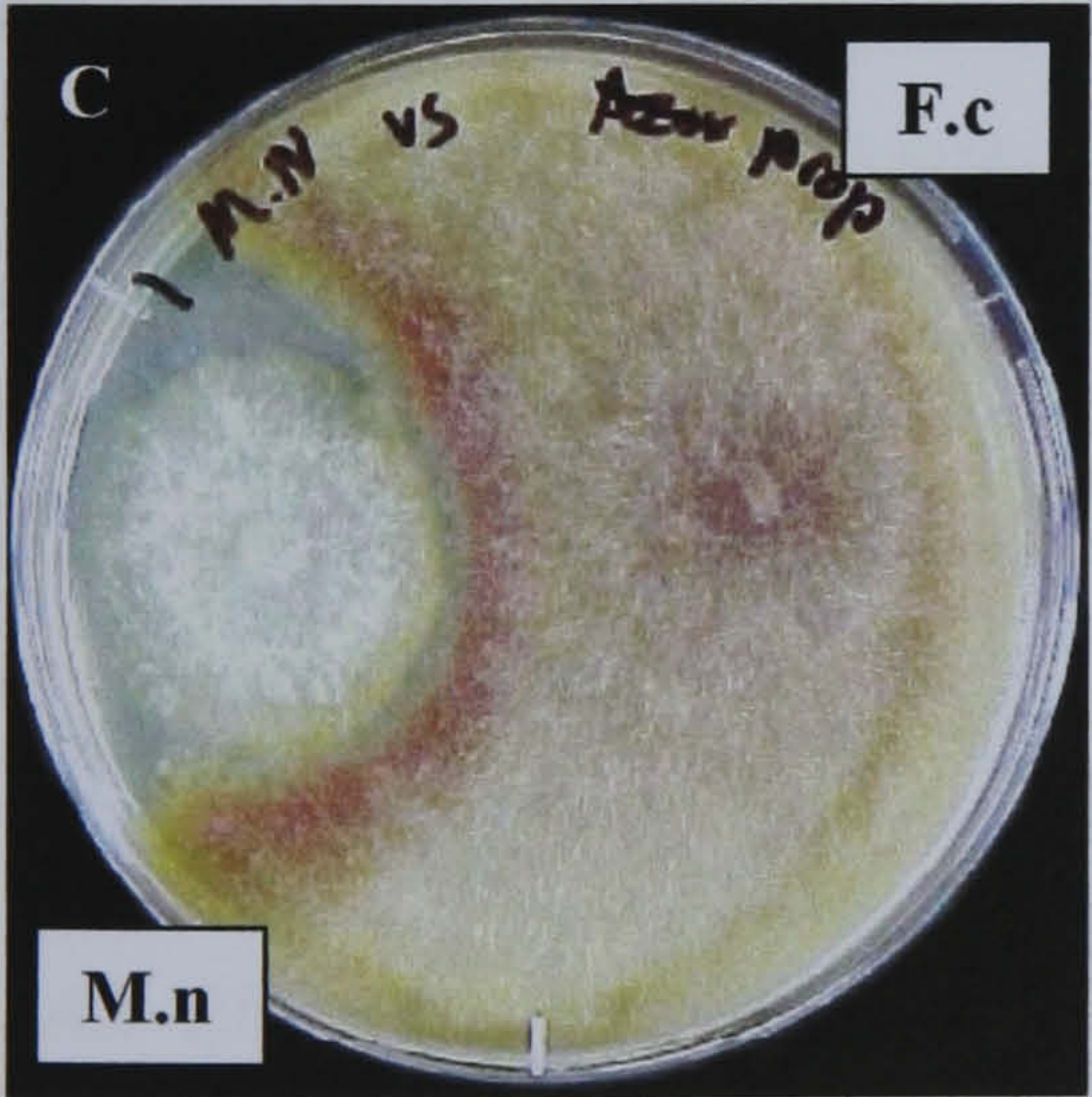
**Type of interaction**  
Mutual intermingling

**Interaction score**  
 $M.n = 1 / F.c = 1$



**Type of interaction**  
Mutual intermingling

**Interaction score**  
 $M.n = 2 / F.c = 2$



**Type of interaction**  
Mutual intermingling

**Interaction score**  
 $M.n = 0 / F.c = 4$

**Plate 3.1** Examples of interactions between *M. nivale* (18/1/N) and *F. culmorum* on wheat agar. A. 0.98  $a_w$  15 °C, B. 0.995  $a_w$  25 °C and C. 0.995  $a_w$  25 °C treated 0.5  $\mu\text{g g}^{-1}$  propioconazole. Species key F.c *F. culmorum* and M.n *M. nivale*.



### 3.4.2 Interaction scores and Indices of Dominance ( $I_D$ )

Tables 3.12 to 3.15 show the interaction scores for *F. culmorum* vs. other common wheat grain fungi, on wheat based media adjusted to various  $a_w$  levels at 15 °C and 25 °C. The first number of the interaction scores always represents that for *F. culmorum*.

#### a). *In vitro*

On wheat agar substrate (Tables 3.12 and 3.13) *F. culmorum* was generally dominant over non-*Fusaria* at 25 °C and all  $a_w$  levels. Interactions with other *Fusaria* resulted in either inhibition on contact or dominance over *F. culmorum*. At 15 °C, *F. culmorum* was generally dominated by the other species at 0.995  $a_w$  and 0.98  $a_w$ . These dominant interactions changed to inhibition on contact or inhibition at a distance under drier conditions (0.955  $a_w$ ). The sum of the Index of Dominance scores indicates that at *F. culmorum* is dominant at 25 °C across all the  $a_w$  levels, tested when grown on wheat agar.

#### b). *In situ*

On irradiated wheat grain substrate (Tables 3.14 and Table 3.15) *F. graminearum* and *F. poae* were dominant over *F. culmorum* at most  $a_w$  levels and temperatures. Where the other *Fusaria* were not dominant over *F. culmorum*, inhibition on contact interactions resulted. Non-*Fusaria* were mostly dominated by *F. culmorum* at both  $a_w$  and temperature levels tested. The sum of the Index of Dominance scores indicates that against the species assayed, *F. culmorum* was dominant on irradiated wheat grain under all the  $a_w$  and temperature conditions, although dominance was achieved by a smaller margin at 0.95  $a_w$  and 15 °C.

### 3.4.3 Effect of interactions on growth of *F. culmorum*

Figure 3.22 shows the relative growth rates of *F. culmorum* on irradiated wheat grain when grown in homogenous and dual culture, at two  $a_w$  levels and temperatures. Growth rates of *F. culmorum* were unaffected by the presence of *C. herbarum*, *F. graminearum*, *F. poae*, *M. nivale* and *M. nivale* var. *majus* at 0.955  $a_w$  and 25 °C.

Under the same conditions *F. culmorum*'s growth rate was stimulated when grown in dual culture with *A. tenuissima* and *P. verrucosum*. *C. herbarum*, *M. nivale* var. *majus* and *P. verrucosum* stimulated growth of *F. culmorum* 0.955  $a_w$  and 15°C and *M. nivale* at both 0.995  $a_w$  and 0.955  $a_w$ , and 15 °C. At 0.995  $a_w$  and 25 °C, growth of *F. culmorum* was inhibited by all of the species except *C. herbarum* and *P. verrucosum* which did not significantly change growth rates and *A. tenuissima* which caused a stimulation in growth rate.



**Table 3.12** Interaction and Index of Dominance ( $I_D$ ) scores for *F. culmorum* vs. various wheat grain field and storage fungi. Substrate wheat agar at three  $a_w$  levels incubation 25 °C.

$a_w$ /spp	0.995	0.98	0.955	$I_D$
<i>F. graminearum</i>	1/1	0/4	2/2	3/7
<i>F. poae</i>	2/2	2/2	2/2	6/6
<i>A. tenuissima</i>	4/0	4/0	4/0	12/0
<i>C. herbarum</i>	4/0	4/0	4/0	12/0
<i>M. nivale</i>	2/2	4/0	4/0	10/2
<i>M. nivale var majus</i>	4/0	4/0	4/0	12/0
<i>P. verrucosum</i>	4/0	4/0	2/2	10/2
<b>Total (<math>I_D</math>)</b>	<b>21/5</b>	<b>22/6</b>	<b>22/6</b>	<b>65/17</b>

**Table 3.13** Interaction and Index of Dominance ( $I_D$ ) scores for *F. culmorum* vs. various wheat grain field and storage fungi. Substrate wheat agar at three  $a_w$  levels incubation 15 °C.

$a_w$ /spp	0.995	0.98	0.955	$I_D$
<i>F. graminearum</i>	0/4	0/4	2/2	2/10
<i>F. poae</i>	2/2	1/1	2/2	5/5
<i>A. tenuissima</i>	0/4	0/4	2/2	2/10
<i>C. herbarum</i>	2/2	4/0	2/2	8/4
<i>M. nivale</i>	0/4	1/1	3/3	4/8
<i>M. nivale var majus</i>	0/4	0/4	2/2	2/10
<i>P. verrucosum</i>	2/2	4/0	2/2	8/4
<b>Total (<math>I_D</math>)</b>	<b>6/22</b>	<b>10/16</b>	<b>15/15</b>	<b>31/53</b>

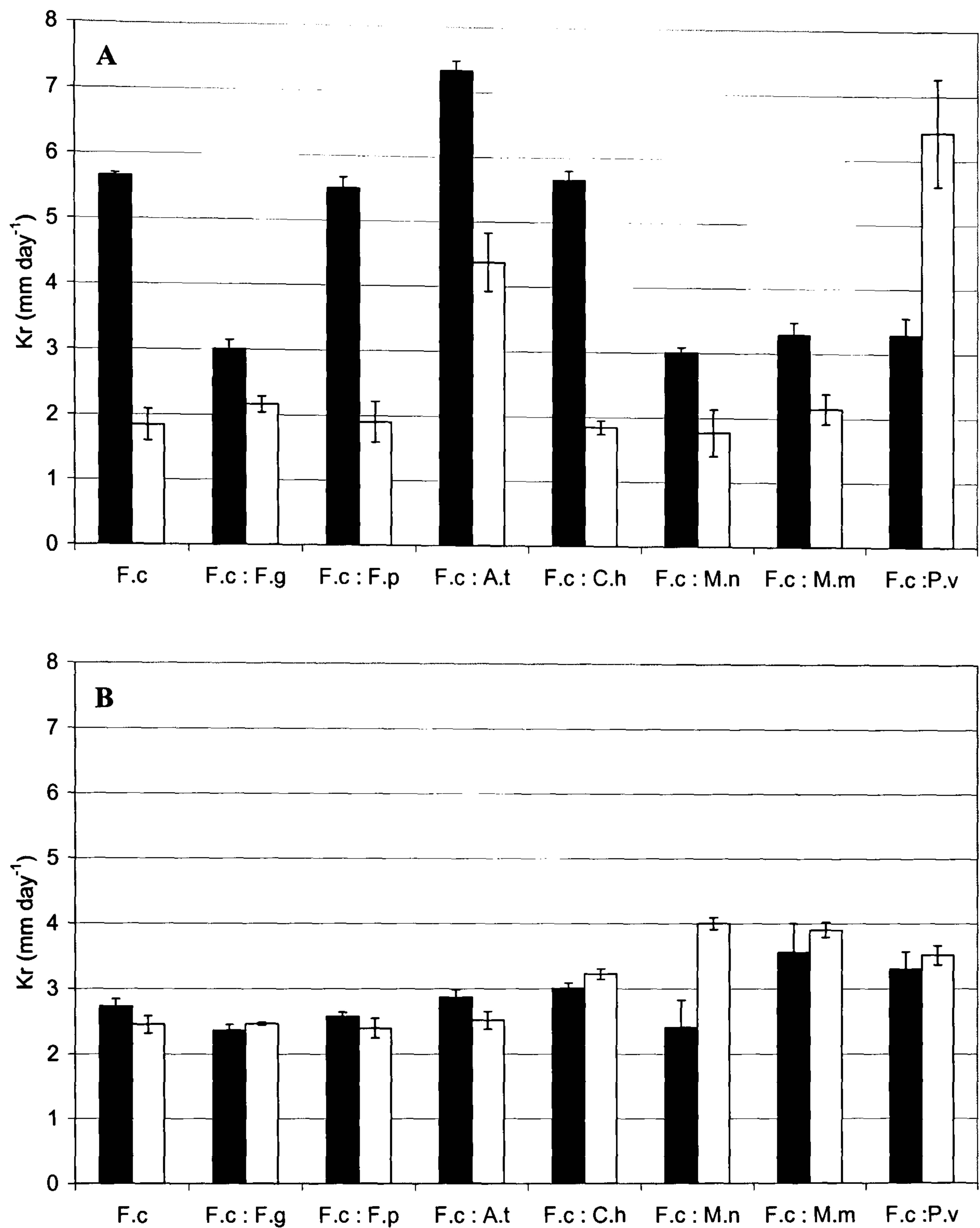
**Table 3.14** Interaction and Index of Dominance ( $I_D$ ) scores for *F. culmorum* vs. various wheat grain field and storage fungi. Substrate irradiated wheat grain at two  $a_w$  levels incubation 25 °C.

$a_w$ /spp	0.995	0.95	$I_D$
<i>F. graminearum</i>	0/4	0/4	0/8
<i>F. poae</i>	1/1	2/2	3/3
<i>A. tenuissima</i>	4/0	4/0	8/0
<i>C. herbarum</i>	4/0	4/0	8/0
<i>M. nivale</i>	4/0	4/0	8/0
<i>M. nivale var majus</i>	4/0	4/0	8/0
<i>P. verrucosum</i>	2/2	4/0	6/2
<b>Total (<math>I_D</math>)</b>	<b>19/7</b>	<b>22/6</b>	<b>31/13</b>

**Table 3.15** Interaction and Index of Dominance ( $I_D$ ) scores for *F. culmorum* vs. various wheat grain field and storage fungi. Substrate irradiated wheat grain at two  $a_w$  levels incubation 15 °C.

$a_w$ /spp	0.995	0.95	$I_D$
<i>F. graminearum</i>	0/4	0/4	0/8
<i>F. poae</i>	2/2	0/4	2/6
<i>A. tenuissima</i>	4/0	4/0	8/0
<i>C. herbarum</i>	4/0	4/0	8/0
<i>M. nivale</i>	2/2	4/0	6/2
<i>M. nivale var majus</i>	2/2	0/4	2/6
<i>P. verrucosum</i>	4/0	2/2	6/2
<b>Total (<math>I_D</math>)</b>	<b>18/10</b>	<b>14/14</b>	<b>32/24</b>





**Figure 3.22** Radial extension rates of *F. culmorum* in homogenous and dual culture incubated at 25 °C (A) and 15 °C (B) on a monolayer of irradiated wheat grain ■ 0.995  $a_w$  □ 0.955  $a_w$ . Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale* var. *majus* and P.v *P. verrucosum*. Bars represent standard error.

### 3.4.4 Effect of interactions between *F. culmorum* and other spoilage fungi on DON /NIV production

#### a). *In vitro*

Figures 3.23 and 3.24 show DON and NIV production by *F. culmorum* on wheat agar adjusted to 0.995  $a_w$ , 0.98  $a_w$  and 0.955  $a_w$  at 25 °C and 15 °C respectively. DON production was stimulated when *F. culmorum* was cultured with *P. verrucosum* at 0.99  $a_w$  and 0.98  $a_w$ . No DON was detected in dual culture plates of *F. graminearum* and *M. nivale*. DON was not detected in dual culture plates adjusted to 0.98  $a_w$  or below containing *F. poae* or *C. herbarum*.

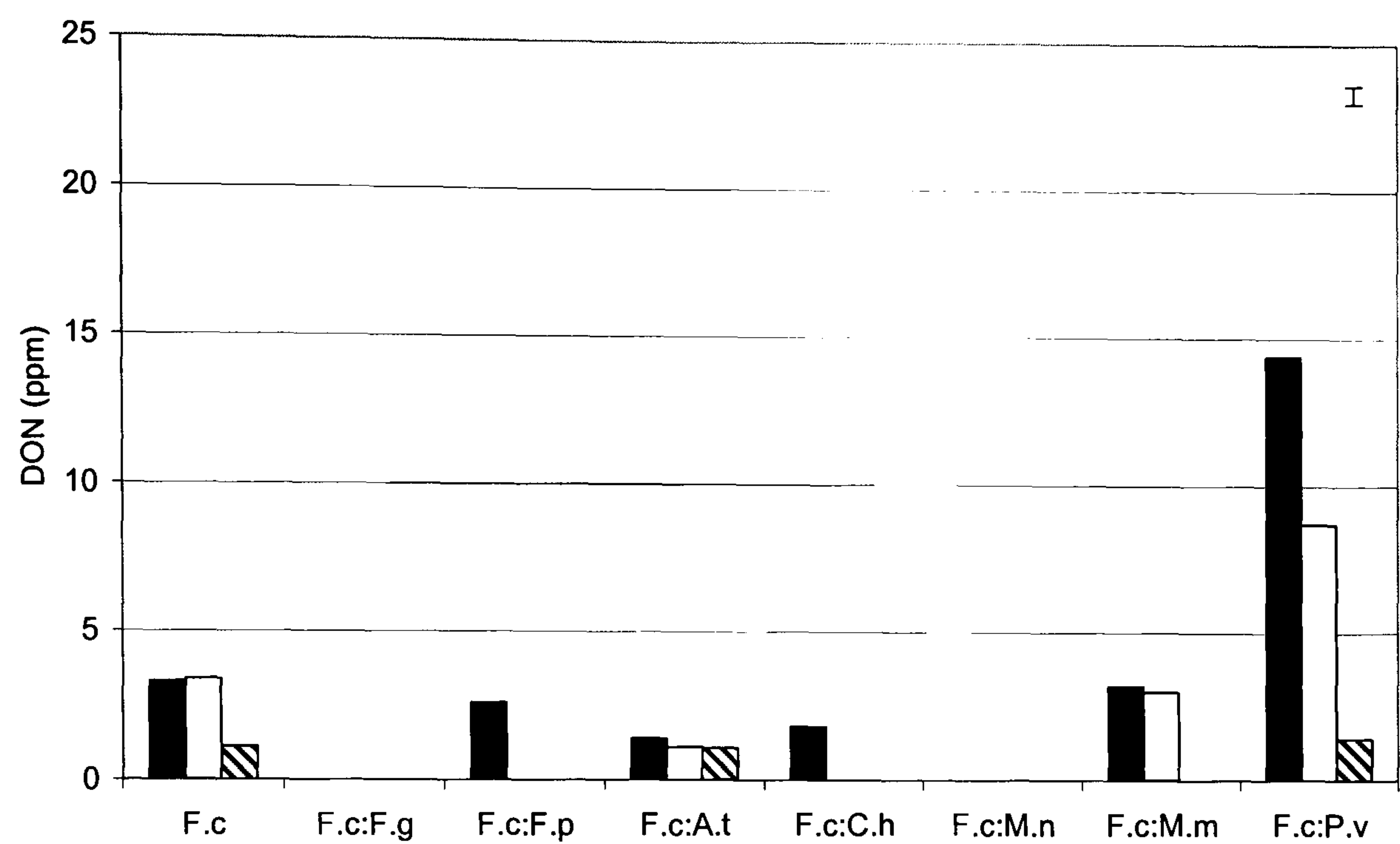
Large amounts of NIV were seen in dual culture plates at 0.98  $a_w$  and 0.99  $a_w$  containing *F. poae*. All of the dual culture plates at 0.98  $a_w$  apart from those containing *F. poae* had low or zero NIV. Table 3.16 summarises the effects of interactions on DON and NIV levels on wheat agar under the  $a_w$  levels tested.

#### b). *In situ*

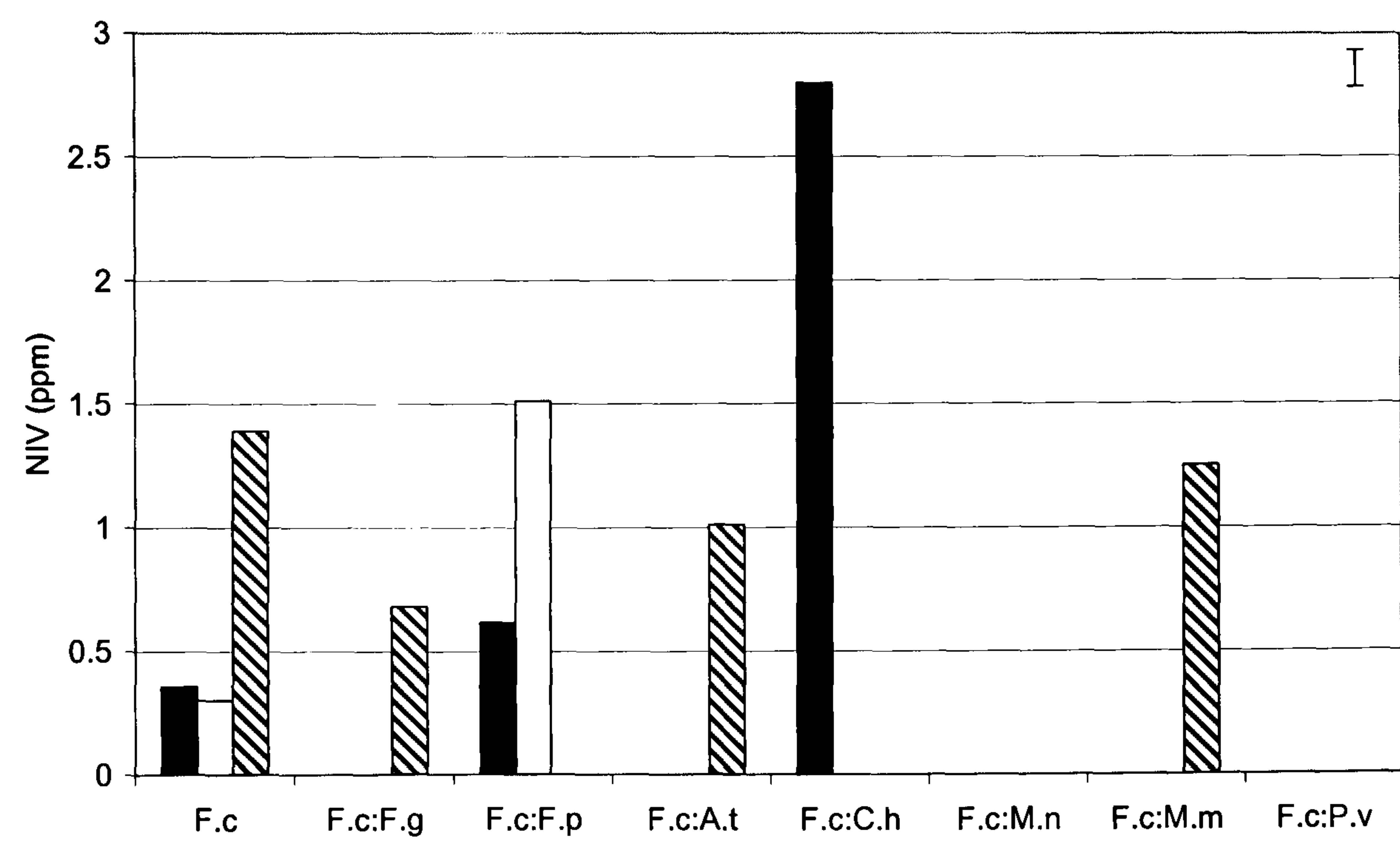
Figures 3.25 to 3.28 show the DON and NIV levels obtained from homogenous and dual *F. culmorum* cultures grown on irradiated wheat grain at two  $a_w$  levels and temperatures. DON production was stimulated at 25 °C 0.955  $a_w$  (Figure 3.25) when *F. culmorum* was grown in the presence of *C. herbarum*, *F. graminearum*, *M. nivale* and *P. verrucosum*. At 15 °C incubation (Figure 3.26) DON production was stimulated in the 0.955  $a_w$  *F. graminearum* dual culture plates. Inhibition of DON occurred at the same temperature but at 0.995  $a_w$  in dual cultures containing *C. herbarum*, *F. graminearum*, and *M. nivale*. *A. tenuissima* and *M. nivale* var. *majus* inhibited DON production at both  $a_w$  levels.

NIV levels were increased at 25 °C 0.995  $a_w$  (Figure 3.27) by *C. herbarum* and at both  $a_w$  levels by *A. tenuissima*. At 15 °C incubation (Figure 3.28) *F. graminearum* and *M. nivale* var. *majus* stimulated NIV when the  $a_w$  level was 0.955  $a_w$ . All of the species inhibited NIV production by *F. culmorum* at 0.995  $a_w$  15 °C. Table 3.17 summarises the effects of interactions on DON and NIV levels on wheat grain under the  $a_w$  levels tested.





**Figure 3.23** DON production in homogenous and dual culture on wheat agar interaction plates adjusted to ■ 0.995 a<sub>w</sub>, □ 0.980 a<sub>w</sub>, and ▨ 0.955 a<sub>w</sub>. The plates were incubated at 25 °C.



**Figure 3.24** NIV production in homogenous and dual culture on wheat agar interaction plates adjusted to ■ 0.995 a<sub>w</sub>, □ 0.980 a<sub>w</sub>, and ▨ 0.955 a<sub>w</sub>. The plates were incubated at 25 °C.

**Table 3.16** Effect of interactions on *F. culmorum* DON and NIV production under various a<sub>w</sub> levels, on wheat agar at 25°C. a). DON b). NIV. NS, not significantly different to control. ↑ ↓ significant increase or decrease respectively in mycotoxin levels.

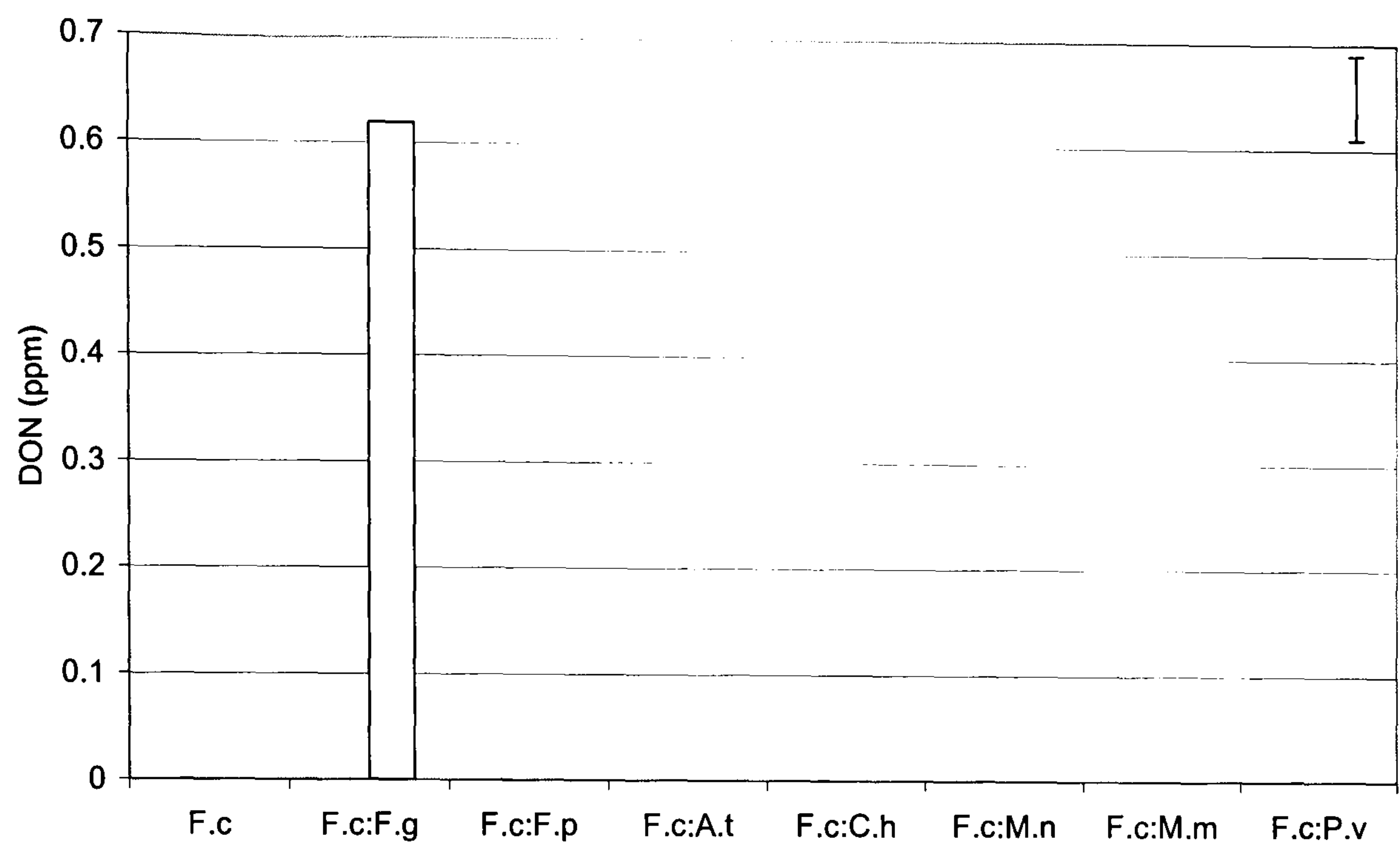
a).

	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>
Fc:Fg	↓	↓	↓
Fc:Fp	NS	↓	↓
Fc:At	↓	↓	NS
Fc:Ch	↓	↓	↓
Fc:Mn	↓	↓	↓
Fc:Mm	NS	NS	↓
Fc:Pv	↑	↑	NS

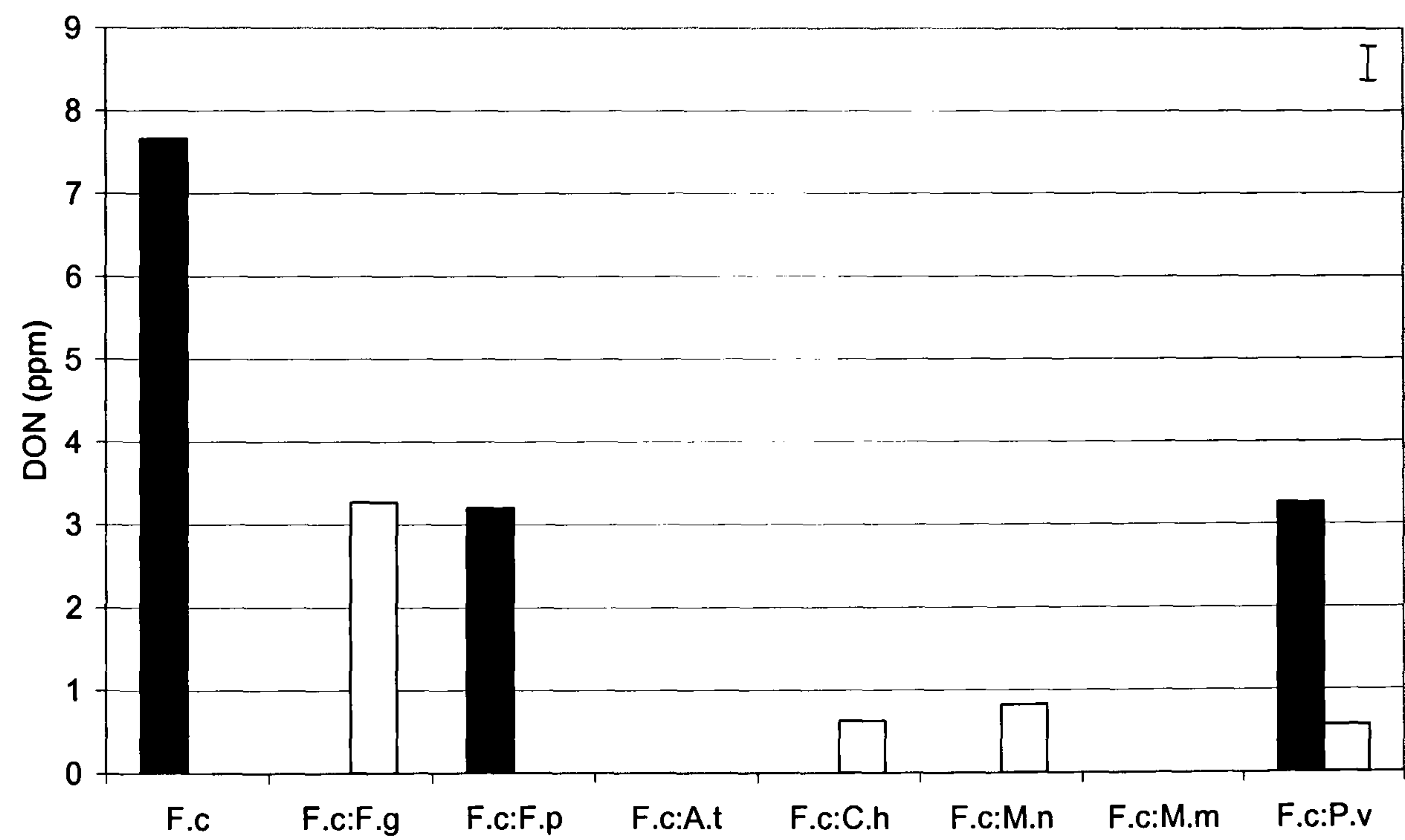
b).

	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>
Fc:Fg	↓	↓	↓
Fc:Fp	↑	↑	↓
Fc:At	↓	↓	↓
Fc:Ch	↑	↓	↓
Fc:Mn	↓	↓	↓
Fc:Mm	↓	↓	NS
Fc:Pv	↓	↓	↓

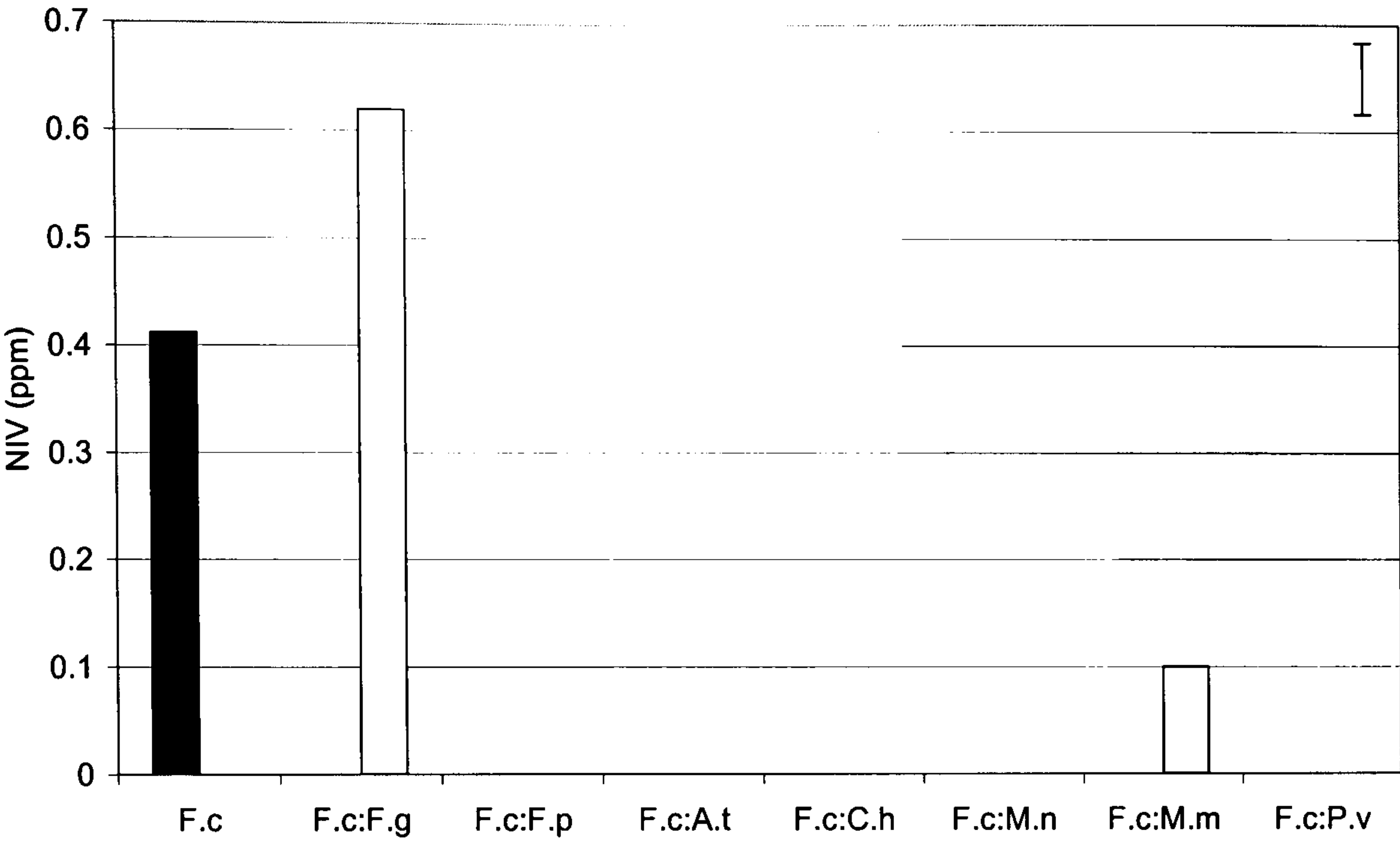




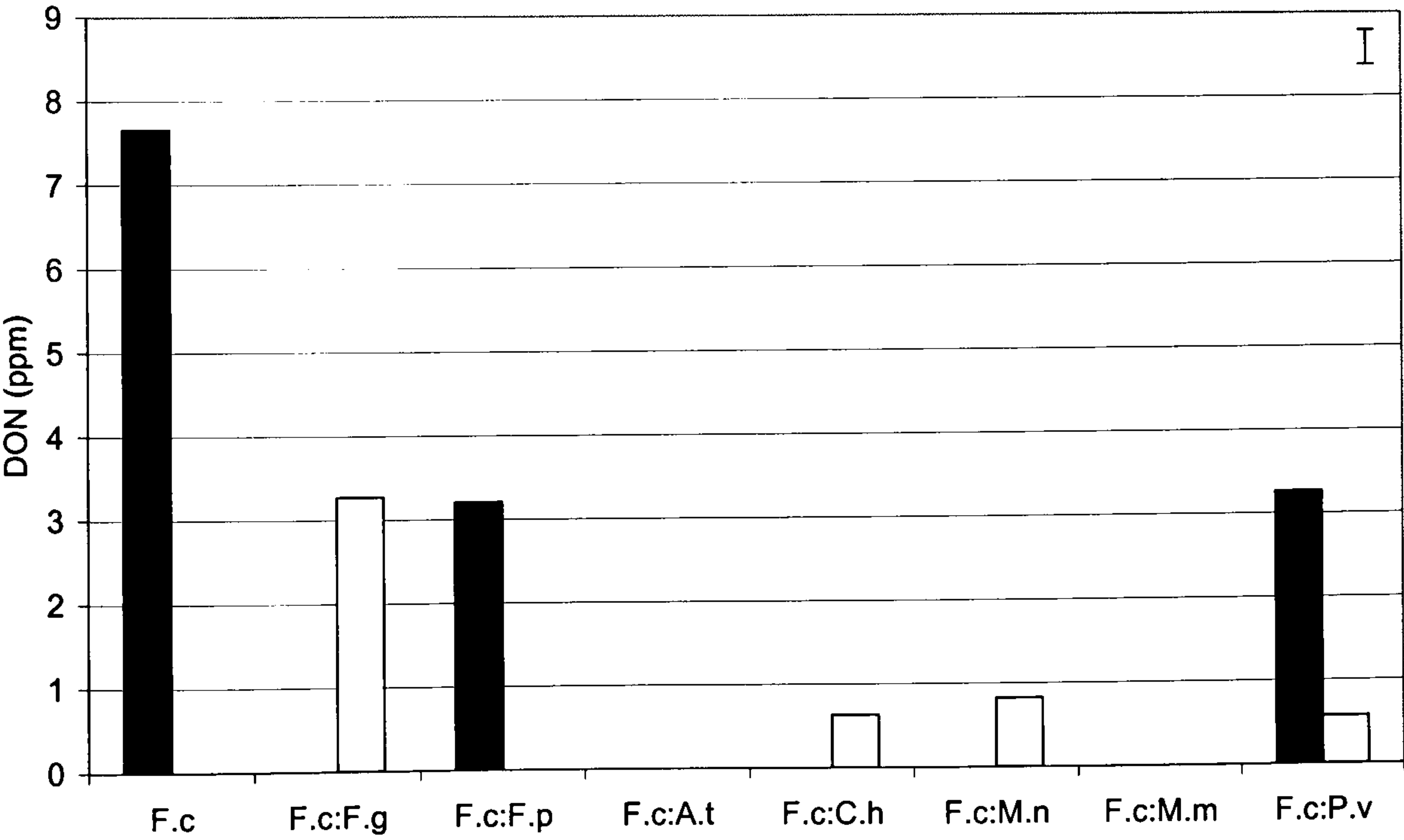
**Figure 3.25** DON production in homogenous and dual culture on irradiated wheat grain interaction plates adjusted to ■ 0.995  $a_w$ , and □ 0.955  $a_w$ . The plates were incubated at 15 °C. Bar represents LSD.



**Figure 3.26** DON production in homogenous and dual culture on irradiated wheat grain interaction plates adjusted to ■ 0.995  $a_w$ , and □ 0.955  $a_w$ . The plates were incubated at 25 °C. Bar represents LSD.



**Figure 3.27** NIV production in homogenous and dual culture on irradiated wheat grain interaction plates adjusted to ■ 0.995 a<sub>w</sub>, and □ 0.955 a<sub>w</sub>. The plates were incubated at 15 °C. Bar represents LSD.



**Figure 3.28** NIV production in homogenous and dual culture on irradiated wheat grain interaction plates adjusted to ■ 0.995 a<sub>w</sub>, and □ 0.955 a<sub>w</sub>. The plates were incubated at 25 °C. Bar represents LSD.



**Table 3.17** Effect of interactions on *F. culmorum* DON and NIV production under various  $a_w$  levels, on wheat grain at 15 and 25°C. a). DON b). NIV. NS, not significantly different to control. ↑ ↓ significant increase or decrease respectively in mycotoxin levels.

a).

	15°C		25°C	
	0.995 $a_w$	0.955 $a_w$	0.995 $a_w$	0.955 $a_w$
Fc:Fg	NS	↑	↓	↑
Fc:Fp	NS	NS	↓	NS
Fc:At	NS	NS	↓	NS
Fc:Ch	NS	NS	↓	↑
Fc:Mn	NS	NS	↓	↑
Fc:Mm	NS	NS	↓	NS
Fc:Pv	NS	NS	↓	↑

b).

	15°C		25°C	
	0.995 $a_w$	0.955 $a_w$	0.995 $a_w$	0.955 $a_w$
Fc:Fg	↓	↑	NS	NS
Fc:Fp	↓	NS	NS	NS
Fc:At	↓	NS	↑	↑
Fc:Ch	↓	NS	↑	NS
Fc:Mn	↓	NS	NS	NS
Fc:Mm	↓	↑	NS	NS
Fc:Pv	↓	NS	NS	NS

### 3.5 IMPACT OF SUBOPTIMAL FUNGICIDE CONCENTRATIONS ON INTERACTIONS AND DON/NIV PRODUCTION BY *F. CULMORUM*

Building upon the previous interaction experiments this study introduces fungicides as an additional stress factor. Wheat agar and irradiated wheat grain that had been adjusted to different  $a_w$  levels was treated with low concentration of commonly used cereal fungicides to simulate fungicide residues found in stored grain. These substrates were then inoculated with *F. culmorum* either homogenously or in dual culture with other wheat grain contaminant fungi.

#### 3.5.1 Interactions and Index of Dominance scores on wheat agar and irradiated wheat grain treated with low levels of cereal fungicides

Table 3.18 and Table 3.19 show the interactions and  $I_D$  scores for *F. culmorum* against the seven other fungi on wheat agar at 15 and 25 °C respectively. Against the non-*Fusaria* with the exception of *P. verrucosum*, *F. culmorum* was dominant upon contact at most of the  $a_w$  levels and 25 °C. *P. verrucosum* was dominated by *F. culmorum* at 0.995  $a_w$  and 0.98  $a_w$  in fungicide treatments of propiconazole or epoxiconazole. At 0.955  $a_w$  25 °C with both these fungicides mutual inhibition on contact resulted. The same interaction result was obtained in treatments with azoxystrobin regardless of  $a_w$  level. When incubated at 15 °C, *F. culmorum* was unable to compete as effectively as at 25 °C. Many conditions when *F. culmorum* was competitive at 25 °C changed to mutual inhibition on contact or at a distance at 15 °C. *F. culmorum* interactions with the *Microdochium* species resulted in the most dramatic changes due to temperature with *M. nivale* and *M. nivale* var. *majus* able to dominate or inhibit *F. culmorum*.

On irradiated wheat grain (Table 3.20 and Table 3.21) interactions between *F. culmorum* and other fungi were similar to those on agar. *F. culmorum* generally dominated the non-*Fusaria* at 25 °C changing to inhibition on contact at 15 °C. *F. graminearum* was able to dominate *F. culmorum* on contact at 25 °C and all of the  $a_w$  levels, regardless of the fungicide treatment. At 15 °C *F. graminearum* vs. *F.*



*culmorum* interactions mostly resulted in mutual inhibition on contact. The only exception to this was at 0.95  $a_w$  in the epoxiconazole, treatment.

**Table 3.18** Interactions between *F. culmorum* and other common wheat grain fungi, on wheat agar at various  $a_w$  levels and containing  $0.5\ \mu\text{g g}^{-1}$  of a commercial fungicide. Plates were incubated at 25 °C.

Aw/spp	<u>Azoxystrobin</u>			<u>Propioconazole</u>			<u>Epoxiconazole</u>			I <sub>D</sub>
	0.995	0.98	0.95	0.995	0.98	0.95	0.995	0.98	0.95	
F.g	1/1	0/4	2/2	1/1	0/4	2/2	1/1	0/4	2/2	9/21
F.p	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	18/18
A.t	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	36/0
C.h	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	36/0
M.n	4/0	4/0	4/0	2/2	4/0	4/0	4/0	4/0	4/0	34/2
M.m	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	4/0	36/0
P.v	2/2	2/2	2/2	4/0	4/0	2/2	4/0	4/0	2/2	26/10
Total	21/5	20/8	22/6	21/5	22/6	22/6	23/3	22/6	22/6	195/51

**Table 3.19** Interactions between *F. culmorum* and other common wheat grain fungi, on wheat agar at various  $a_w$  levels and containing  $0.5\ \mu\text{g g}^{-1}$  of a commercial fungicide. Plates were incubated at 15 °C.

Aw/spp	<u>Azoxystrobin</u>			<u>Propioconazole</u>			<u>Epoxiconazole</u>			I <sub>D</sub>
	0.995	0.98	0.95	0.995	0.98	0.95	0.995	0.98	0.95	
F.g	0/4	0/4	2/2	0/4	0/4	0/4	0/4	0/4	2/2	4/30
F.p	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/1	2/2	17/17
A.t	0/4	2/2	2/2	0/4	2/2	2/2	0/4	0/4	2/2	10/26
C.h	2/2	2/2	3/3	2/2	2/2	2/2	2/2	4/0	2/2	19/17
M.n	0/4	0/4	3/3	0/4	0/4	3/3	0/4	0/4	3/3	9/33
M.m	0/4	0/4	3/3	0/4	0/4	3/3	0/4	0/4	2/2	8/32
P.v	3/3	4/0	2/2	2/2	4/0	2/2	3/3	4/0	2/2	26/14
Total	9/23	10/18	17/17	6/22	10/18	14//18	7/23	9/17	15/15	97/171



**Table 3.20** Interactions between *F. culmorum* and other common wheat grain niche fungi, on irradiated wheat grain at two  $a_w$  levels and containing  $0.5 \mu\text{g g}^{-1}$  of a commercial fungicide. Plates were incubated at 25 °C.

	<u>Azoxystrobin</u>		<u>Propioconazole</u>		<u>Epoxiconazole</u>		<b>I<sub>D</sub></b>
Aw/spp	0.995	0.95	0.995	0.95	0.995	0.95	
F.g	0/4	0/4	0/4	0/4	0/4	0/4	<b>0/24</b>
F.p	1/1	2/2	1/1	2/2	1/1	2/2	<b>9/9</b>
A.t	4/0	4/0	4/0	4/0	4/0	4/0	<b>24/0</b>
C.h	4/0	4/0	4/0	4/0	4/0	4/0	<b>24/0</b>
M.n	4/0	4/0	4/0	4/0	4/0	4/0	<b>24/0</b>
M.m	4/0	4/0	4/0	4/0	4/0	4/0	<b>24/0</b>
P.v	2/2	4/0	2/2	4/0	2/2	4/0	<b>18/6</b>
<b>Total</b>	<b>19/7</b>	<b>22/6</b>	<b>19/7</b>	<b>22/6</b>	<b>19/7</b>	<b>22/6</b>	<b>123/39</b>

**Table 3.21** Interactions between *F. culmorum* and other common wheat grain niche fungi, on irradiated wheat grain at two  $a_w$  levels and containing  $0.5 \mu\text{g g}^{-1}$  of a commercial fungicide. Plates were incubated at 15 °C.

	<u>Azoxystrobin</u>		<u>Propioconazole</u>		<u>Epoxiconazole</u>		<b>I<sub>D</sub></b>
Aw/spp	0.995	0.95	0.995	0.95	0.995	0.95	
F.g	2/2	2/2	2/2	2/2	2/2	0/4	<b>10/14</b>
F.p	2/2	0/4	2/2	0/4	2/2	2/2	<b>8/16</b>
A.t	4/0	4/0	4/0	4/0	4/0	4/0	<b>24/0</b>
C.h	4/0	4/0	4/0	4/0	4/0	4/0	<b>24/0</b>
M.n	2/2	2/2	2/2	4/0	4/0	4/0	<b>18/6</b>
M.m	1/1	2/2	4/0	2/2	4/0	2/2	<b>15/7</b>
P.v	2/2	2/2	2/2	2/2	4/0	2/2	<b>14/10</b>
<b>Total</b>	<b>17/9</b>	<b>16/16</b>	<b>20/8</b>	<b>18/10</b>	<b>24/4</b>	<b>18/10</b>	<b>113/57</b>

### 3.5.2 Effect of interacting species on the growth rate of *F. culmorum* on irradiated wheat grain in the presence of sub-optimal concentrations of cereal fungicides

Figure 3.29 shows the effect of interacting fungi on *F. culmorum* growth on an irradiated wheat grain substrate treated with  $0.5 \mu\text{g g}^{-1}$  azoxystrobin. In conditions with freely available water ( $0.995 a_w$ ) at  $25^\circ\text{C}$  growth of *F. culmorum* was greatly stimulated by the presence of *F. graminearum* or *A. tenuissima*. Inhibition of growth occurred under the same conditions in dual culture with *M. nivale*, *M. nivale* var. *majus* and *P. verrucosum*. At the same temperature but at  $0.955 a_w$ , growth of *F. culmorum* was only affected significantly by *A. tenuissima* and *P. verrucosum*, resulting in an increase in *F. culmorum* growth. At  $15^\circ\text{C}$  there was little difference in growth rates of *F. culmorum* in dual cultures to that of the homogenous controls. However, there was some stimulation of growth in dual cultures involving *C. herbarum*, *M. nivale*, *M. nivale* var. *majus* and *P. verrucosum*. This stimulation occurred at both  $a_w$  levels although it was more pronounced at  $0.955 a_w$  with *M. nivale*, *M. nivale* var. *majus* and *P. verrucosum*.

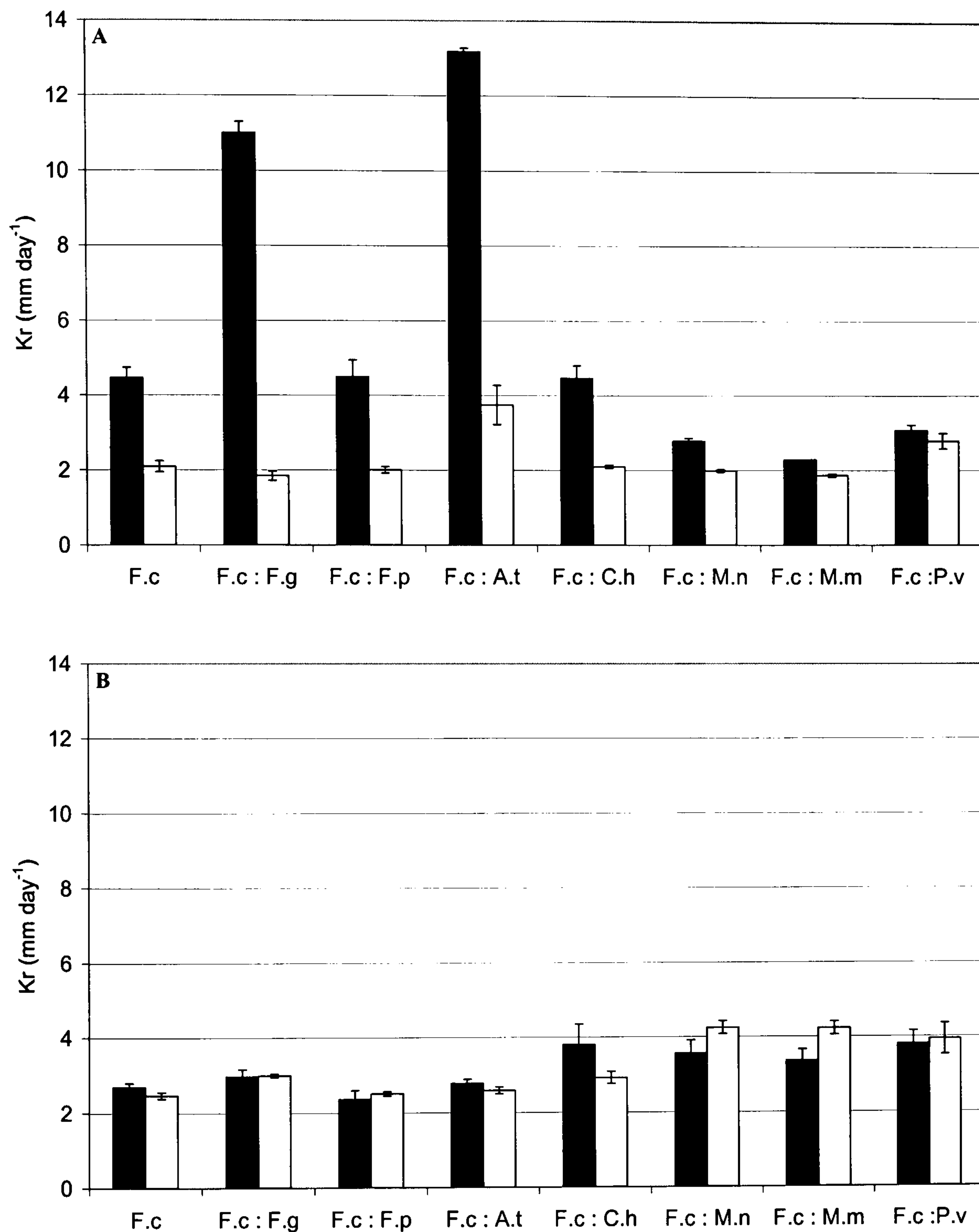
Figure 3.30 shows the effect of competition between *F. culmorum* and other species on growth of *F. culmorum* in grain treated with  $0.5 \mu\text{g g}^{-1}$  propiconazole. Significant stimulation of *F. culmorum* growth rates occurred in dual culture with *A. tenuissima*, *C. herbarum* and *F. poae* at  $25^\circ\text{C}$  and  $0.995 a_w$ . The other test fungi all suppressed *F. culmorum* growth rates at this condition. Under drier conditions growth of *F. culmorum* was generally unaffected by the presence of other species at  $25^\circ\text{C}$ , except for *P. verrucosum*, which caused a significant stimulation of growth.

At  $15^\circ\text{C}$  growth of *F. culmorum* was stimulated in dual culture with *C. herbarum* and *M. nivale* at  $0.995 a_w$  and *M. nivale*, *M. nivale* var. *majus* and *P. verrucosum* at  $0.955 a_w$ . At this temperature the only inhibition that occurred was in dual culture with *M. nivale* var. *majus* at  $0.995 a_w$ .

Figure 3.30 also shows the effect of interacting fungi on *F. culmorum* growth on an irradiated wheat grain substrate treated with  $0.5 \mu\text{g g}^{-1}$  epoxiconazole. Effects on *F.*

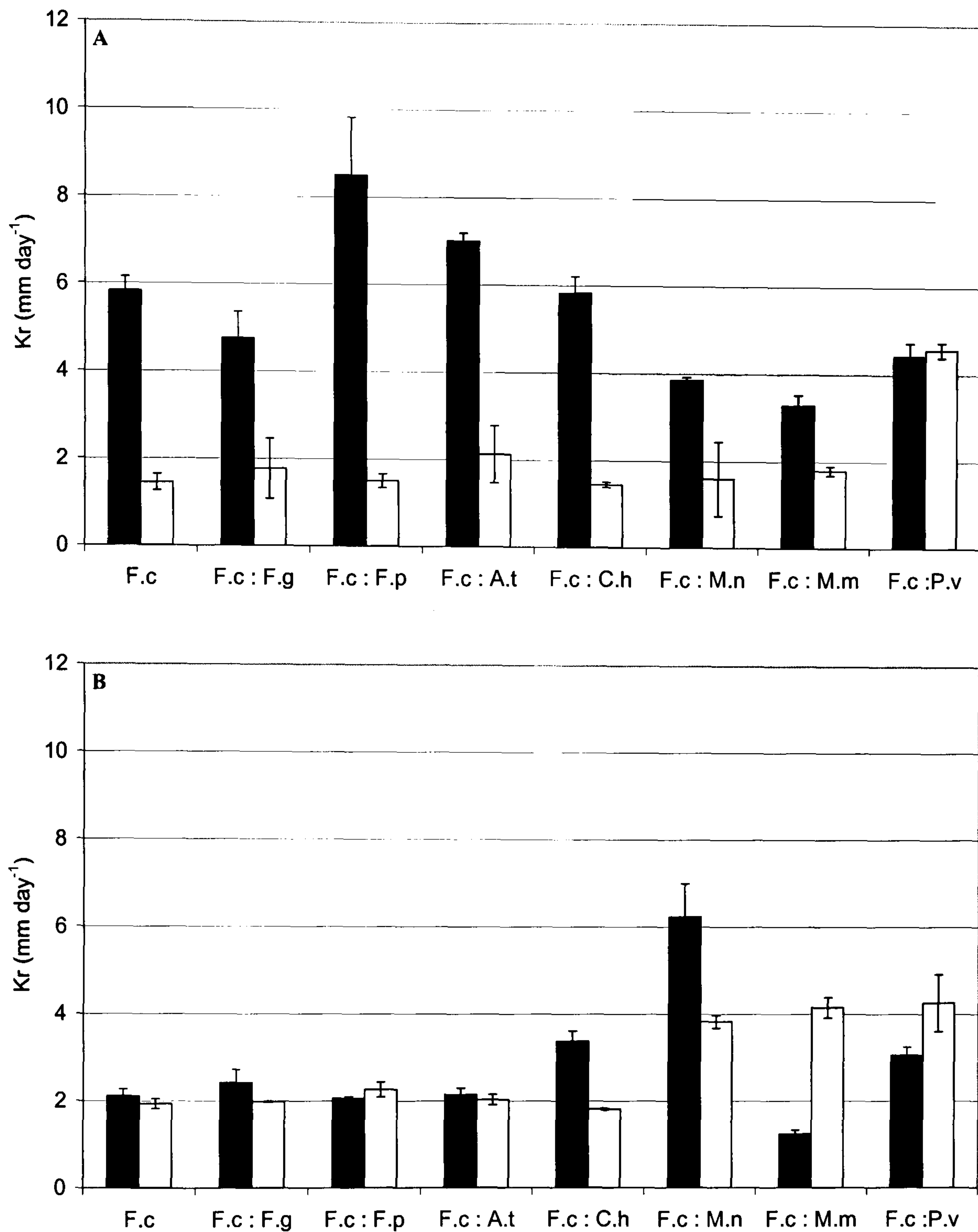


*culmorum* growth rates in dual culture in the presence of epoxiconazole were similar to those observed for the propioconazole treatment. Stimulation and inhibition mainly occurred in the same  $a_w$ , temperature levels and fungal compositions as for the propioconazole treatment. However, the magnitude of the changes in *F. culmorum* growth was greater with propioconazole than epoxiconazole.

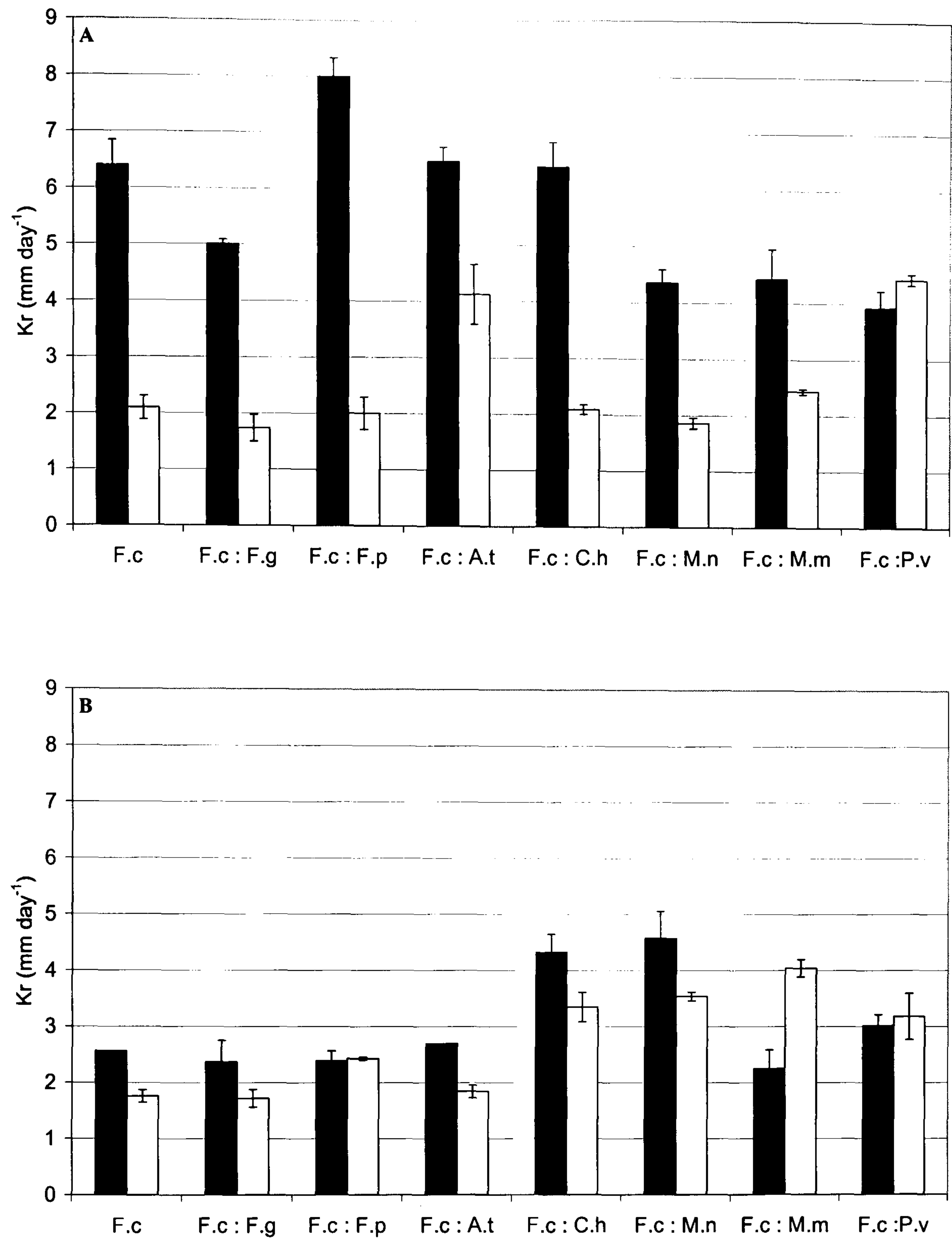


**Figure 3.29** Radial extension rates of *F. culmorum* in homogenous and dual culture incubated at 25 °C (A) and 15 °C (B) on a monolayer of irradiated wheat grain ■ 0.995  $a_w$  □ 0.955  $a_w$  treated with 0.5  $\mu\text{g g}^{-1}$  azoxystrobin. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale* var. *majus* and P.v *P. verrucosum*. Bars represent standard error.





**Figure 3.30** Radial extension rates of *F. culmorum* in homogenous and dual culture incubated at 25 °C (A) and 15 °C (B) on a monolayer of irradiated wheat grain ■ 0.995  $a_w$  □ 0.955  $a_w$  treated with 0.5  $\mu\text{g g}^{-1}$  propiconazole. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale* var. *majus* and P.v *P. verrucosum*. Bars represent standard error.



**Figure 3.31** Radial extension rates of *F. culmorum* in homogenous and dual culture incubated at 25 °C (A) and 15 °C (B) on a monolayer of irradiated wheat grain ■ 0.995 a<sub>w</sub> □ 0.955 a<sub>w</sub> treated with 0.5 µg g<sup>-1</sup> epoxiconazole. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale* var. *majus* and P.v *P. verrucosum*. Bars represent standard error.



### 3.5.3 Effect of interactions and sub-optimal concentrations of fungicides on DON and NIV production

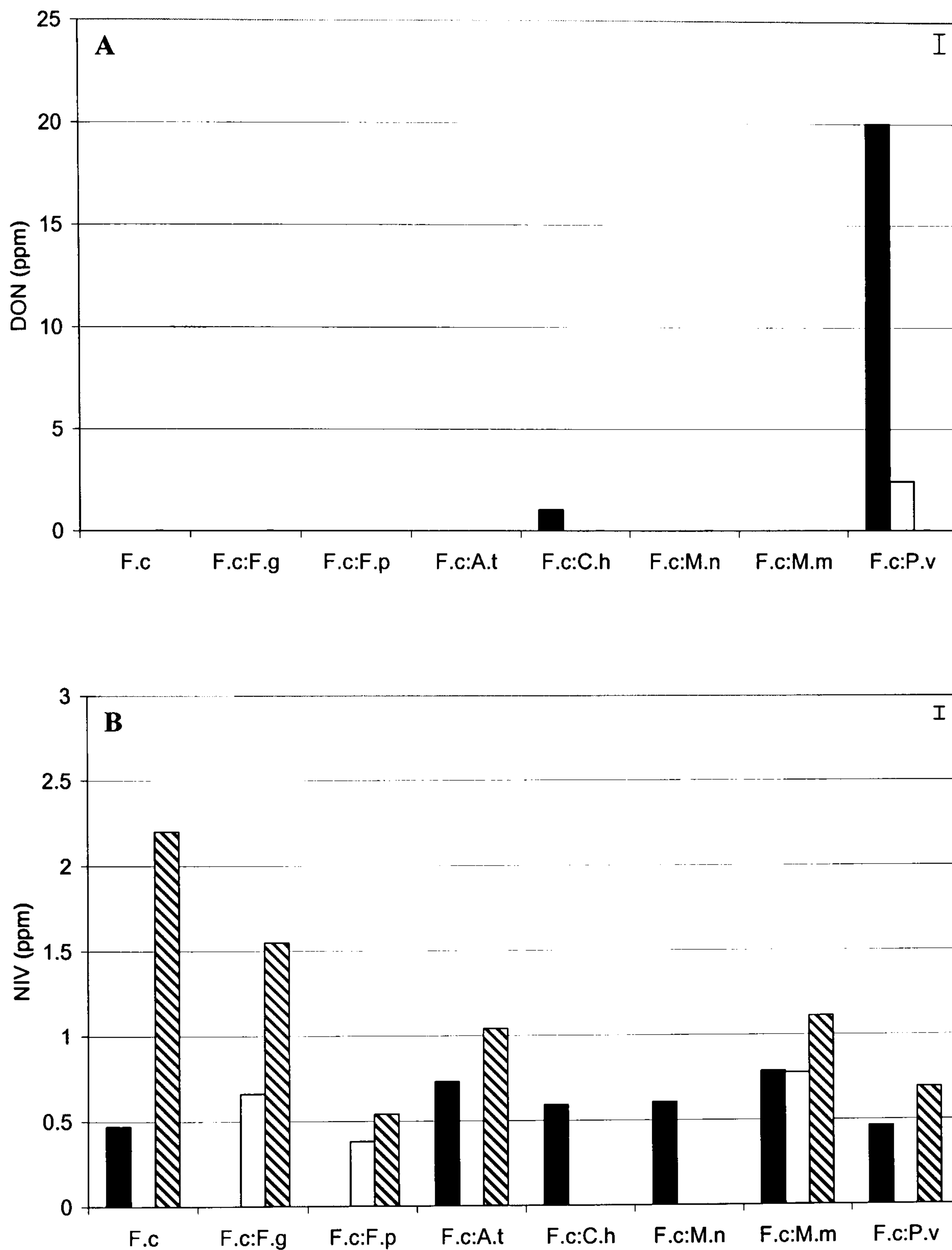
Figures 3.32 - 3.34 show the DON and NIV levels obtained in homogenous and dual culture on wheat agar incorporating  $0.5 \mu\text{g g}^{-1}$  of a common fungicide adjusted to three  $a_w$  levels and incubated at 25 °C.

#### a). *In vitro*

In the azoxystrobin treatment (Figure 3.32) DON production was stimulated by *C. herbarum* at 0.995  $a_w$  and *P. verrucosum* at both 0.995  $a_w$  and 0.98  $a_w$ . The azoxystrobin completely suppressed DON production in the remaining conditions. NIV production was stimulated at 0.995  $a_w$  by interactions with *A. tenuissima*, *C. herbarum*, *M. nivale* and *M. nivale* var. *majus*. At 0.98  $a_w$  *C. herbarum*, *F. graminearum*, and *F. poae* stimulated the production of NIV by *F. culmorum*. All other conditions either resulted in a reduction or complete inhibition of NIV.

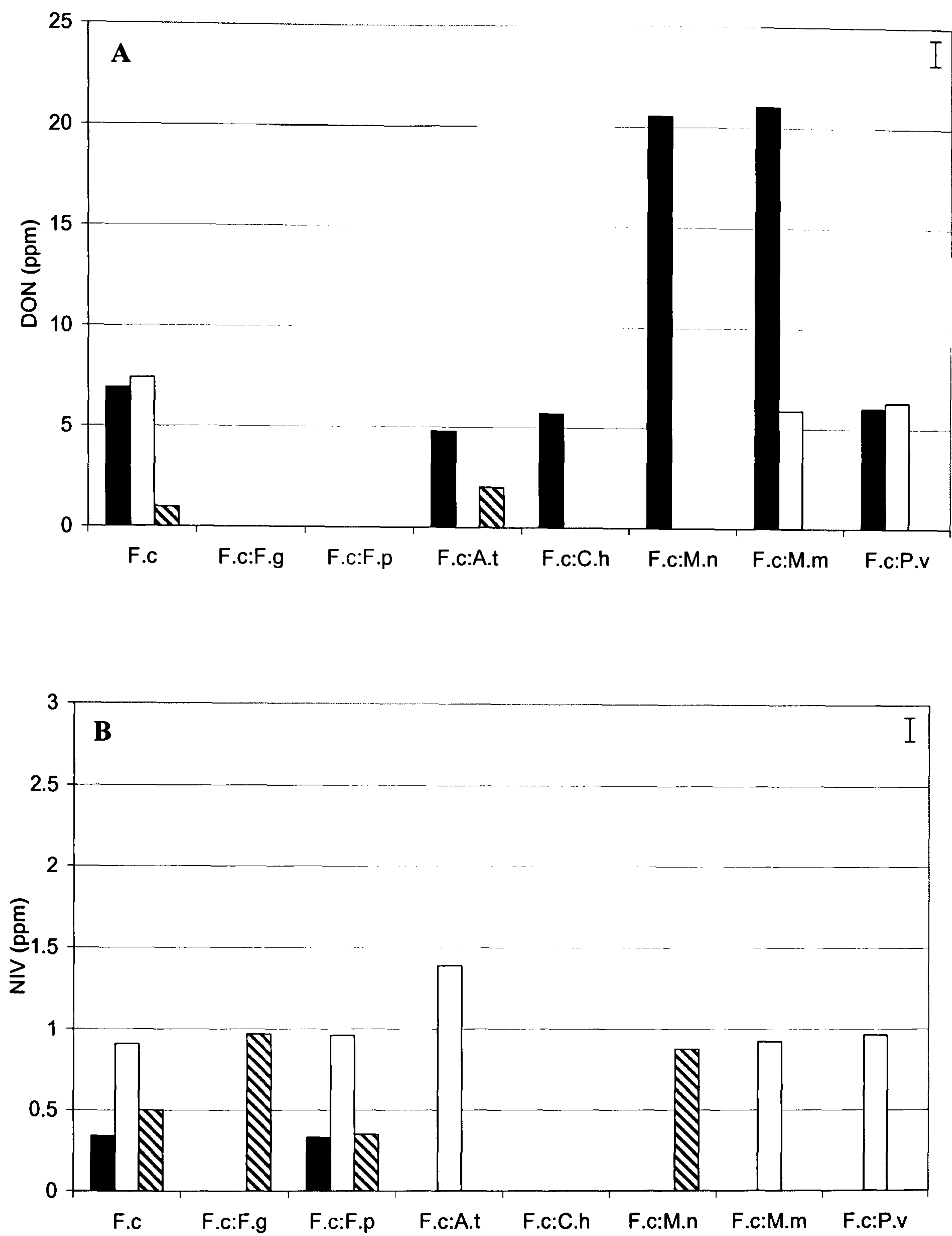
In the dual culture plates treated with propiconazole (Figure 3.33) *M. nivale* and *M. nivale* var. *majus* stimulated DON production by *F. culmorum* at 0.995  $a_w$ . Under the rest of the conditions tested DON production was reduced or inhibited. NIV production by *F. culmorum* was stimulated at 0.98  $a_w$  by *A. tenuissima* and at 0.955  $a_w$  by *F. graminearum* and *M. nivale*. NIV was either unaffected or inhibited under the remaining conditions.

Figure 3.34 shows the DON and NIV results obtained from the wheat agar plates treated with epoxiconazole. The epoxiconazole caused a large increase in DON production in the homogenous cultures at 0.995 $a_w$  and 0.98 $a_w$ . DON production was stimulated at 0.955 $a_w$  in dual cultures with *A. tenuissima*, *M. nivale* var. *majus* and *P. verrucosum*. DON was inhibited under all of the remaining conditions. NIV was stimulated by *F. graminearum* at 0.98  $a_w$  and *M. nivale* at 0.995  $a_w$ . All the remaining conditions either did not affect NIV levels or reduced production by *F. culmorum*. The significant effects of interactions between *F. culmorum* and other species on DON/NIV levels on wheat agar treated with sub-optimal levels of a fungicide are summarised in Table 3.22 (DON) and Table 3.23 (NIV).

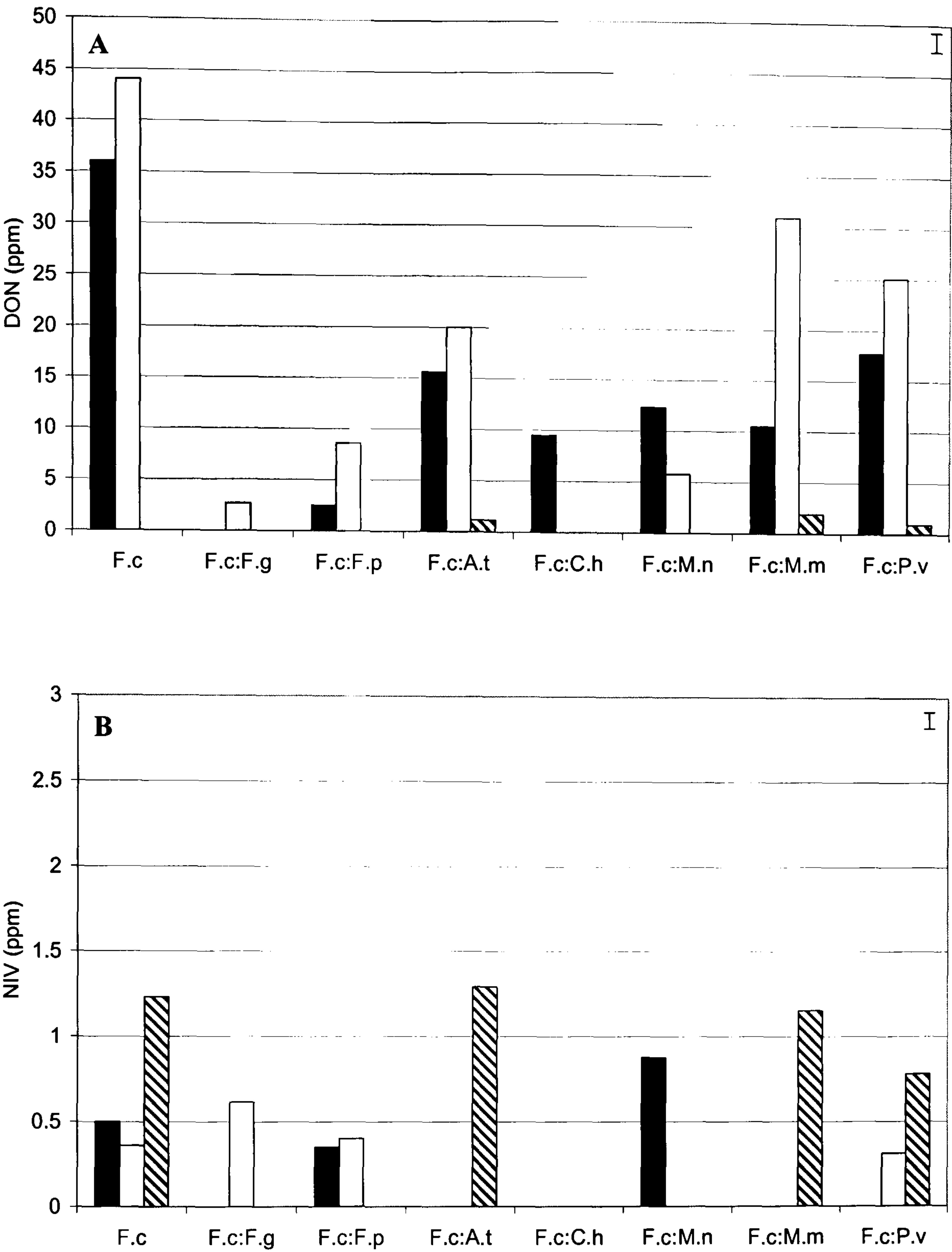


**Figure 3.32** DON (A) and NIV (B) production in homogenous and dual culture on wheat agar plates at various  $a_w$  levels (■ 0.995  $a_w$ , □ 0.980  $a_w$ , and ▨ 0.955  $a_w$ ) and containing  $0.5 \mu\text{g g}^{-1}$  azoxystrobin. Plates were incubated at 25 °C. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale* var. *majus* and P.v *P. verrucosum*. Bar represents LSD.





**Figure 3.33** DON (A) and NIV (B) production in homogenous and dual culture on wheat agar plates at various  $a_w$  levels (■ 0.995  $a_w$ , □ 0.980  $a_w$ , and ▨ 0.955  $a_w$ ) and containing 0.5  $\mu\text{g g}^{-1}$  propiconazole. Plates were incubated at 25 °C. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale var. majus* and P.v *P. verrucosum*. Bar represents LSD.



**Figure 3.34** DON (A) and NIV (B) production in homogenous and dual culture on wheat agar plates at various  $a_w$  levels (■ 0.995  $a_w$ , □ 0.980  $a_w$ , and ▨ 0.955  $a_w$ ) and containing 0.5  $\mu\text{g g}^{-1}$  epoxiconazole. Plates were incubated at 25 °C. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale* var. *majus* and P.v *P. verrucosum*. Bar represents LSD.



**Table 3.22** Effect of interactions on *F. culmorum* DON production under various a<sub>w</sub> levels, on wheat agar at 25°C. a). azoxystrobin b). propioconazole c). epoxiconazole. NS, not significantly different to control. ↑ ↓ significant increase or decrease respectively in mycotoxin levels.

a).

	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>
Fc:Fg	NS	NS	NS
Fc:Fp	NS	NS	NS
Fc:At	NS	NS	NS
Fc:Ch	NS	NS	NS
Fc:Mn	NS	NS	NS
Fc:Mm	NS	NS	NS
Fc:Pv	↑	↑	NS

b).

	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>
Fc:Fg	↓	↓	NS
Fc:Fp	↓	↓	NS
Fc:At	↓	↓	NS
Fc:Ch	↓	↓	NS
Fc:Mn	↑	↓	NS
Fc:Mm	↑	NS	NS
Fc:Pv	NS	NS	NS

c).

	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>
Fc:Fg	↓	↓	NS
Fc:Fp	↓	↓	NS
Fc:At	↓	↓	NS
Fc:Ch	↓	↓	NS
Fc:Mn	↓	↓	NS
Fc:Mm	↓	↓	NS
Fc:Pv	↓	↓	NS

**Table 3.23** Effect of interactions on *F. culmorum* NIV production under various  $a_w$  levels, on wheat agar at 25°C. a). azoxystrobin b). propioconazole c). epoxiconazole. NS, not significantly different to control. ↑ ↓ significant increase or decrease respectively in mycotoxin levels.

a).

	0.995 $a_w$	0.98 $a_w$	0.955 $a_w$
Fc:Fg	↓	↑	↓
Fc:Fp	↓	↑	↓
Fc:At	↑	NS	↓
Fc:Ch	↑	NS	↓
Fc:Mn	↑	NS	↓
Fc:Mm	↑	↑	↓
Fc:Pv	NS	NS	↓

b).

	0.995 $a_w$	0.98 $a_w$	0.955 $a_w$
Fc:Fg	↓	↓	NS
Fc:Fp	↓	↓	NS
Fc:At	↓	↓	NS
Fc:Ch	↓	↓	NS
Fc:Mn	↑	↓	NS
Fc:Mm	↑	NS	NS
Fc:Pv	NS	NS	NS

c).

	0.995 $a_w$	0.98 $a_w$	0.955 $a_w$
Fc:Fg	↓	↑	↓
Fc:Fp	NS	NS	↓
Fc:At	↓	↓	NS
Fc:Ch	↓	↓	↓
Fc:Mn	↑	↓	↓
Fc:Mm	↓	NS	NS
Fc:Pv	↓	↓	↓

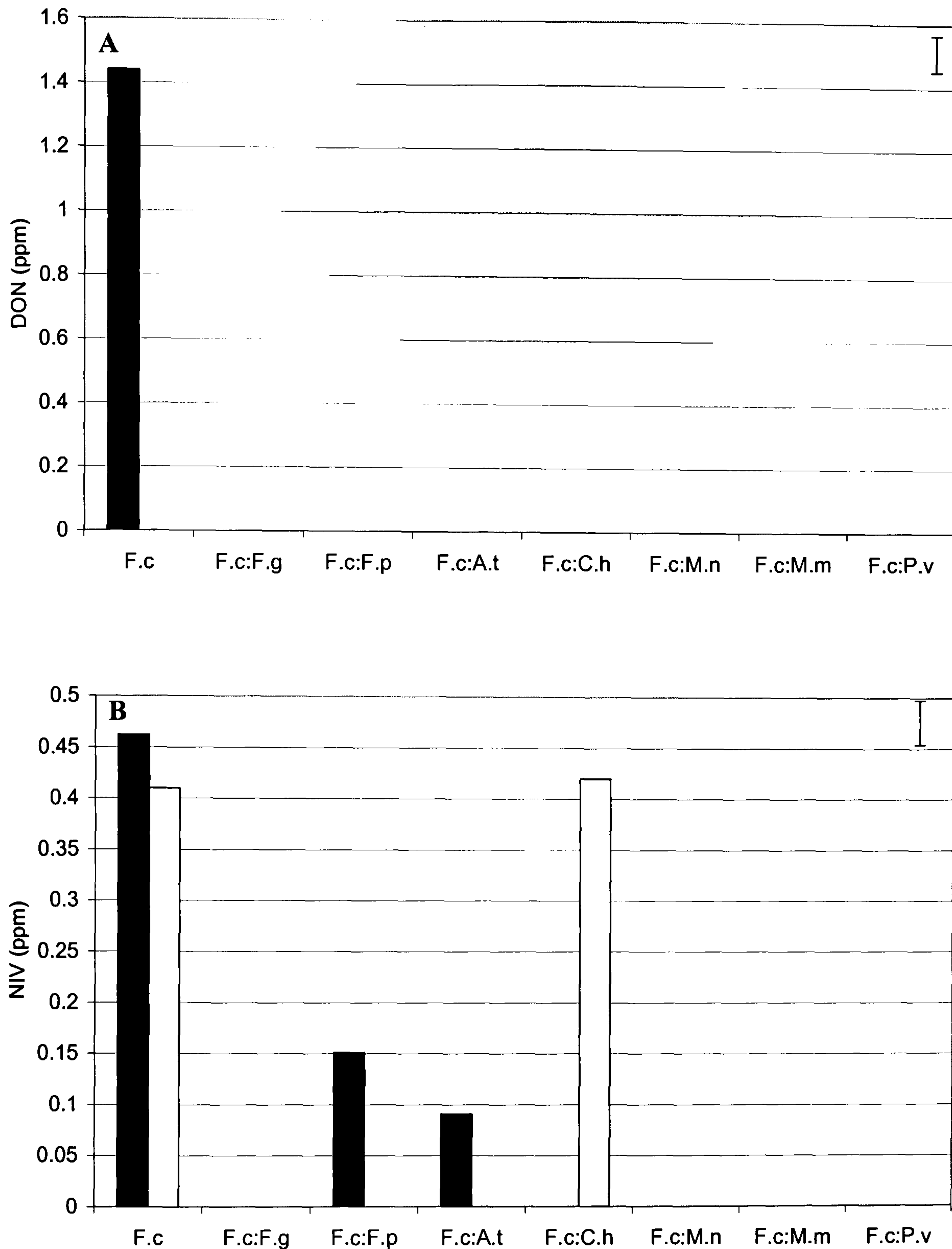


*b). In situ*

Figures 3.35-3.40 show the DON and NIV levels obtained in homogenous and dual culture on irradiated wheat grain incorporating  $0.5 \mu\text{g g}^{-1}$  of a common fungicide adjusted to two  $a_w$  levels and incubated at 25 °C or 15 °C. In the plates treated with azoxystrobin treatments (Figures 3.35 and 3.36) DON production by *F. culmorum* was reduced or inhibited in all of the dual culture plates. Reduction in DON levels was not uniform and varied depending on which species were interacting with *F. culmorum* and the incubation temperature.

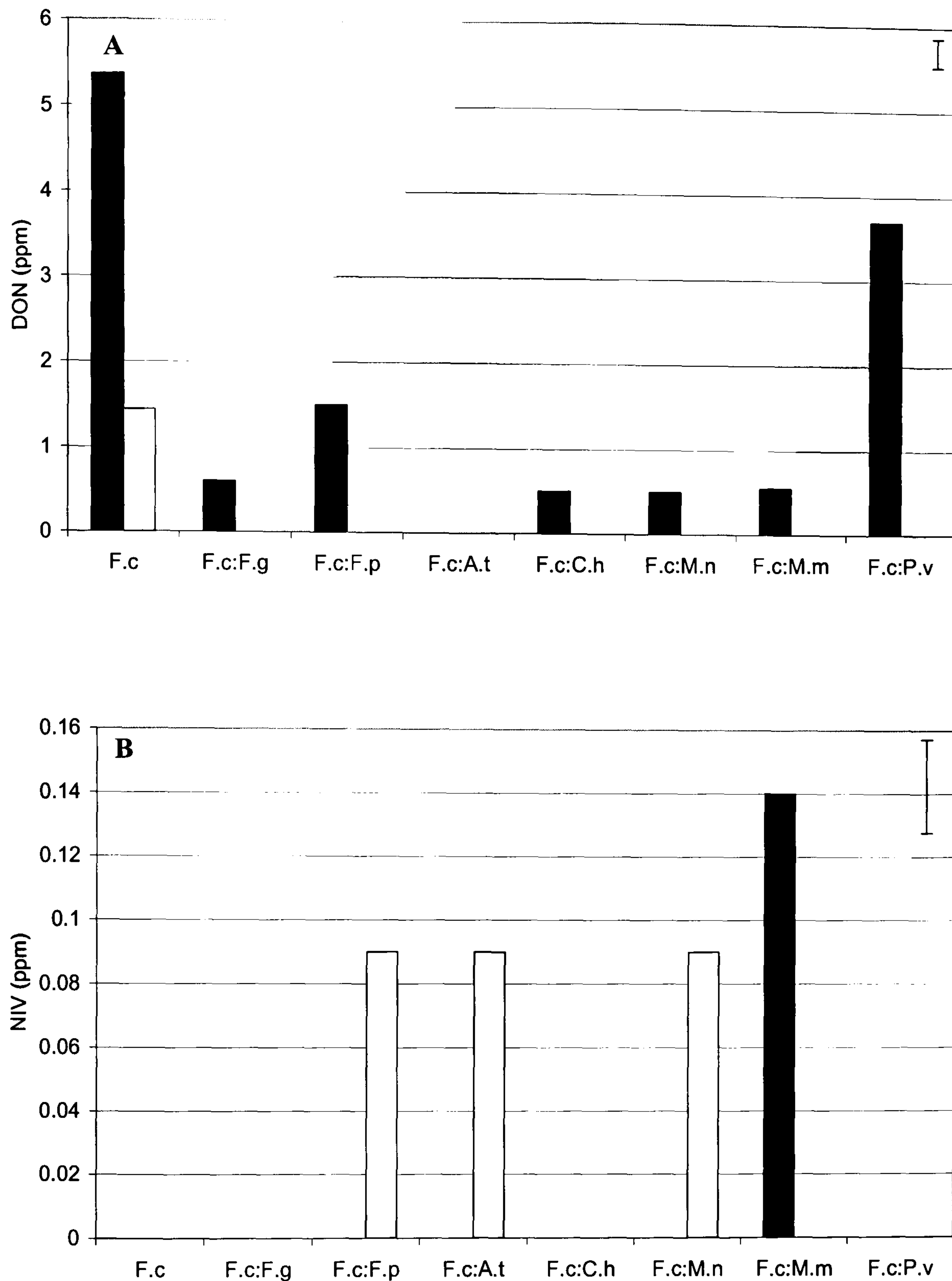
Figures 3.37 and 3.38 show the DON and NIV results for irradiated wheat grain plates treated with propiconazole. Stimulation of DON was caused in dual culture with *F. graminearum* at 0.955  $a_w$  and 25 °C. At 15 °C propiconazole inhibited DON and NIV production in the homogenous cultures. DON production was stimulated at this temperature by *M. nivale* var. *majus* and *P. verrucosum* at 0.955  $a_w$ . NIV was stimulated at 0.995  $a_w$  and 15 °C in dual cultures with *P. verrucosum* and by *M. nivale* var. *majus* and *P. verrucosum* at 0.955  $a_w$  and the same temperature. Under all other conditions NIV production was inhibited.

Figures 3.39 and 3.40 show the DON and NIV results for irradiated wheat grain plates treated with epoxiconazole. In contrast to the stimulation of DON seen in the wheat agar samples, epoxiconazole completely inhibited DON and NIV production by *F. culmorum* on the wheat grain substrate in the homogenous cultures. Stimulation of DON was seen in the following dual cultures: *M. nivale* var. *majus* and *P. verrucosum* 0.995  $a_w$  25 °C, and *C. herbarum* 0.955  $a_w$  15 °C. NIV was stimulated in dual cultures with *M. nivale* var. *majus* 0.955  $a_w$  25 °C and *C. herbarum* and *P. verrucosum* at 0.955  $a_w$ . The significant effects of interactions between *F. culmorum* and other species on DON/NIV levels on wheat grain treated with sub-optimal levels of a fungicide are summarised in Table 3.24 (DON) and Table 3.25 (NIV).

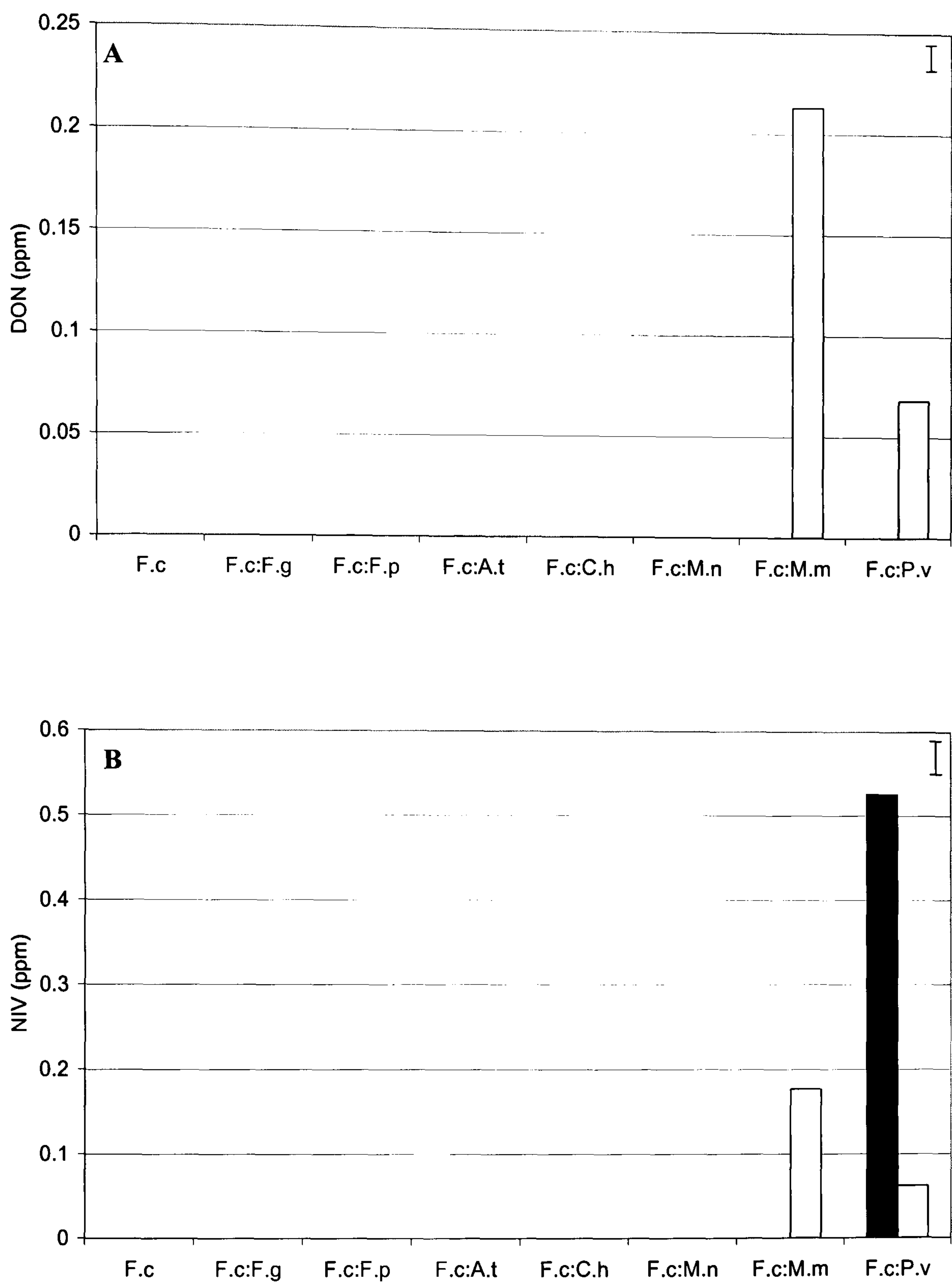


**Figure 3.35** DON (A) and NIV (B) production in homogenous and dual culture on irradiated wheat grain plates at two  $a_w$  levels (■ 0.995  $a_w$ , and □ 0.955  $a_w$ ) and containing  $0.5 \mu\text{g g}^{-1}$  azoxystrobin. Plates were incubated at 15 °C. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale var. majus* and P.v *P. verrucosum*. Bar represents LSD.



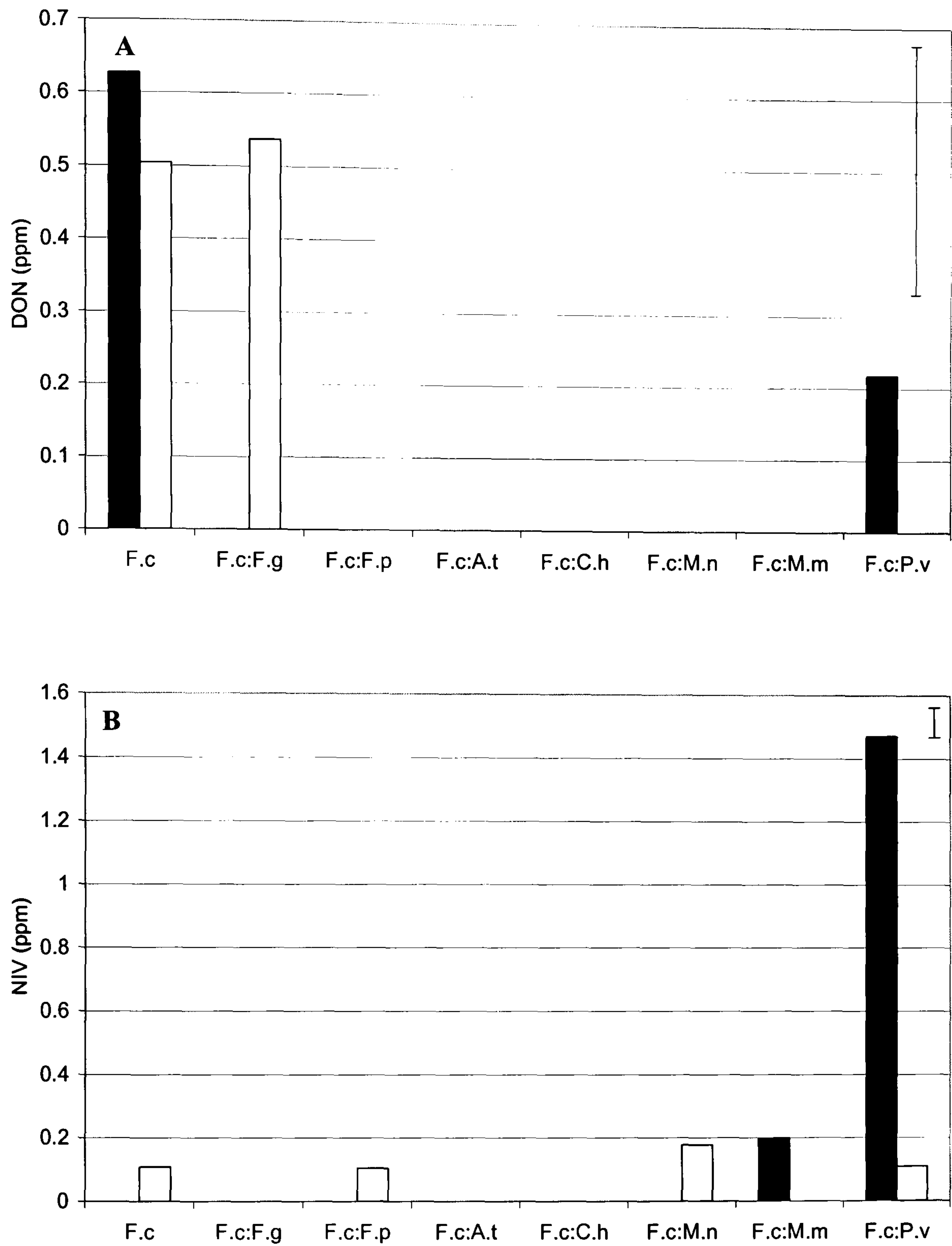


**Figure 3.36** DON (A) and NIV (B) production in homogenous and dual culture on irradiated wheat grain plates at two  $a_w$  levels (■ 0.995  $a_w$ , and □ 0.955  $a_w$ ) and containing  $0.5 \mu\text{g g}^{-1}$  azoxystrobin. Plates were incubated at 25 °C. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale* var. *majus* and P.v *P. verrucosum*. Bar represents LSD.

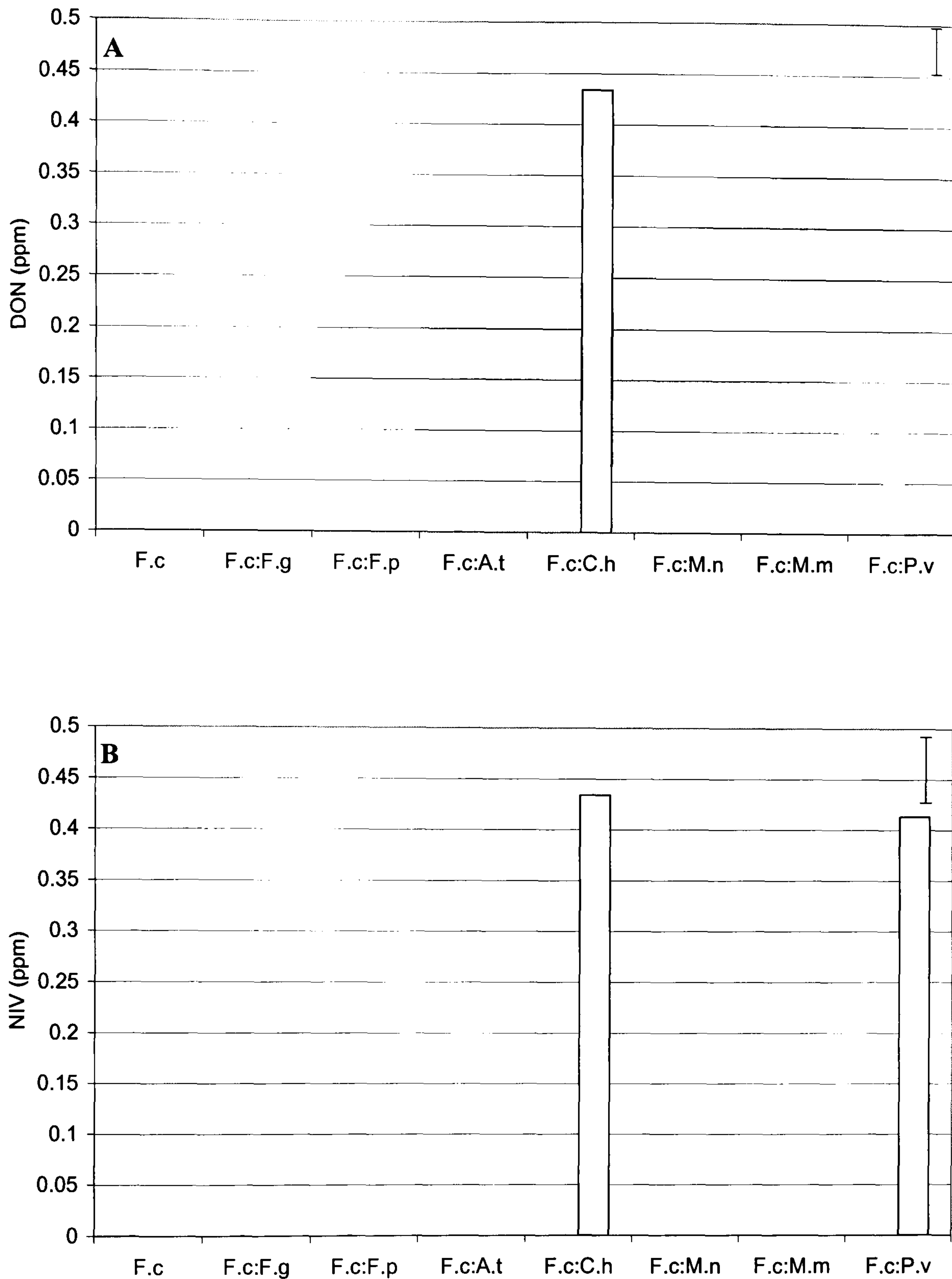


**Figure 3.37** DON (A) and NIV (B) production in homogenous and dual culture on irradiated wheat grain plates at two  $a_w$  levels (■ 0.995  $a_w$ , and □ 0.955  $a_w$ ) and containing 0.5  $\mu\text{g g}^{-1}$  propioconazole. Plates were incubated at 15 °C. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale var. majus* and P.v *P. verrucosum*. Bar represents LSD.



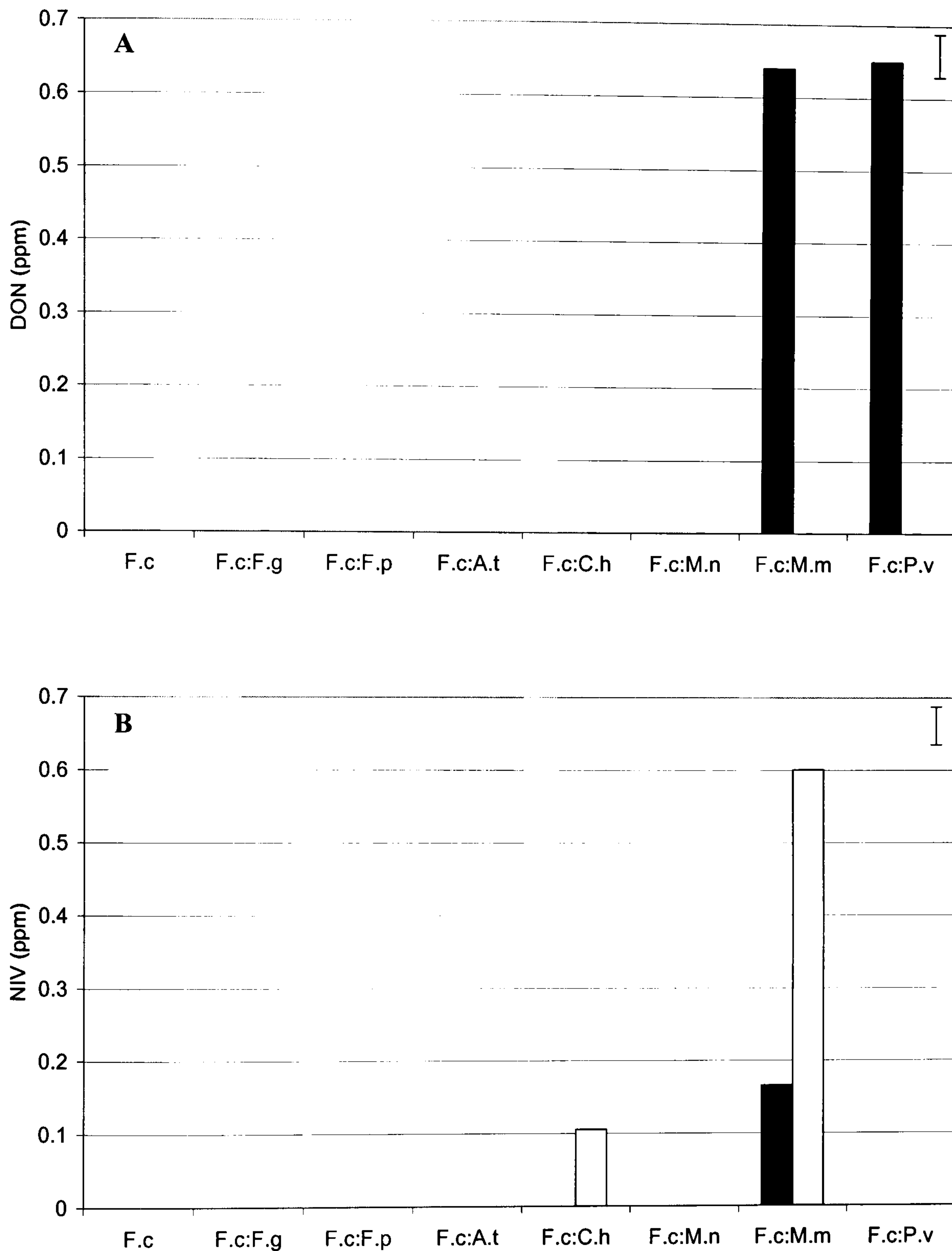


**Figure 3.38** DON (A) and NIV (B) production in homogenous and dual culture on irradiated wheat grain plates at two  $a_w$  levels (■ 0.995  $a_w$ , and □ 0.955  $a_w$ ) and containing 0.5  $\mu\text{g g}^{-1}$  propioconazole. Plates were incubated at 25 °C. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale var. majus* and P.v *P. verrucosum*. Bar represents LSD.



**Figure 3.39** DON (A) and NIV (B) production in homogenous and dual culture on irradiated wheat grain plates at two  $a_w$  levels (■ 0.995  $a_w$ , and □ 0.955  $a_w$ ) and containing  $0.5 \mu\text{g g}^{-1}$  epoxiconazole. Plates were incubated at 15 °C. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum* M.n *M. nivale*, M.m *M. nivale* var. *majus* and P.v *P. verrucosum*. Bar represents LSD.





**Figure 3.40** DON (A) and NIV (B) production in homogenous and dual culture on irradiated wheat grain plates at two  $a_w$  levels (■ 0.995  $a_w$ , and □ 0.955  $a_w$ ) and containing  $0.5 \mu\text{g g}^{-1}$  epoxiconazole. Plates were incubated at 25 °C. Species key F.c *F. culmorum*, F.g *F. graminearum*, F.p *F. poae*, A.t *A. tenuissima*, C.h *C. herbarum*, M.n *M. nivale*, M.m *M. nivale var. majus* and P.v *P. verrucosum*. Bar represents LSD.

**Table 3.24** Effect of interactions on *F. culmorum* DON production under various a<sub>w</sub> levels, and temperatures on wheat grain. a). azoxystrobin b). propioconazole c). epoxiconazole. NS, not significantly different to control. ↑ ↓ significant increase or decrease respectively in mycotoxin levels.

a).

	15°C		25°C	
	0.995 a <sub>w</sub>	0.955 a <sub>w</sub>	0.995 a <sub>w</sub>	0.955a <sub>w</sub>
Fc:Fg	↓	NS	↓	↓
Fc:Fp	↓	NS	↓	↓
Fc:At	↓	NS	↓	↓
Fc:Ch	↓	NS	↓	↓
Fc:Mn	↓	NS	↓	↓
Fc:Mm	↓	NS	↓	↓
Fc:Pv	↓	NS	↓	↓

b).

	15°C		25°C	
	0.995 a <sub>w</sub>	0.955 a <sub>w</sub>	0.995 a <sub>w</sub>	0.955a <sub>w</sub>
Fc:Fg	NS	NS	↓	NS
Fc:Fp	NS	NS	↓	↓
Fc:At	NS	NS	↓	↓
Fc:Ch	NS	NS	↓	↓
Fc:Mn	NS	NS	↓	↓
Fc:Mm	NS	↑	↓	↓
Fc:Pv	NS	↑	↓	↓

c).

	15°C		25°C	
	0.995 a <sub>w</sub>	0.955 a <sub>w</sub>	0.995 a <sub>w</sub>	0.955a <sub>w</sub>
Fc:Fg	NS	NS	NS	NS
Fc:Fp	NS	NS	NS	NS
Fc:At	NS	NS	NS	NS
Fc:Ch	NS	↑	NS	NS
Fc:Mn	NS	NS	NS	NS
Fc:Mm	NS	NS	↑	NS
Fc:Pv	NS	NS	↑	NS



**Table 3.25** Effect of interactions on *F. culmorum* NIV production under various  $a_w$  levels, and temperatures on wheat grain. a). azoxystrobin b). propioconazole c). epoxiconazole. NS, not significantly different to control.  $\uparrow \downarrow$  significant increase or decrease respectively in mycotoxin levels.

a).

	15°C		25°C	
	0.995 $a_w$	0.955 $a_w$	0.995 $a_w$	0.955 $a_w$
Fc:Fg	↓	↓	NS	NS
Fc:Fp	↓	↓	NS	↑
Fc:At	↓	↓	NS	↑
Fc:Ch	↓	NS	NS	NS
Fc:Mn	↓	↓	NS	↑
Fc:Mm	↓	↓	↑	NS
Fc:Pv	↓	↓	NS	NS

b).

	15°C		25°C	
	0.995 $a_w$	0.955 $a_w$	0.995 $a_w$	0.955 $a_w$
Fc:Fg	NS	NS	NS	NS
Fc:Fp	NS	NS	NS	NS
Fc:At	NS	NS	NS	NS
Fc:Ch	NS	NS	NS	NS
Fc:Mn	NS	NS	NS	NS
Fc:Mm	NS	↑	↑	NS
Fc:Pv	↑	↑	↑	NS

c).

	15°C		25°C	
	0.995 $a_w$	0.955 $a_w$	0.995 $a_w$	0.955 $a_w$
Fc:Fg	NS	NS	NS	NS
Fc:Fp	NS	NS	NS	NS
Fc:At	NS	NS	NS	NS
Fc:Ch	NS	↑	NS	↑
Fc:Mn	NS	NS	NS	NS
Fc:Mm	NS	NS	↑	↑
Fc:Pv	NS	↑	NS	NS

### 3.6 CARBON SOURCE UTILIZATION AND NICHE OVERLAP OF COMMON WHEAT GRAIN FUNGI

The carbon source utilization assays were carried out at 0.995 - 93  $a_w$  and incubated at 15 or 25 °C. The assays were used to produce detailed genus and/or species level carbon source utilization profiles for fungi used in the interaction experiments (Section 3.4 ). These profiles were then used to calculate the total number of carbon sources utilized (niche size) and niche overlap index as described in the Methodology (section 2.6 ) for utilization of 95 common carbon sources and 18 key wheat grain carbon sources. It was hypothesised that fungi found to occupy a similar niche would also be those that attempted to exclude or dominate each other in the interaction experiments.

Table 3.26 shows niche size and niche overlap indices for the fungi studied. The target pathogen in all cases is *F. culmorum* (UK3) and thus does not have a niche overlap index (NOI) score. Niche size for the majority of the fungi increased as  $a_w$  level was increased. Temperature elicited a smaller effect on niche size than  $a_w$ . When 95 carbon sources were considered, incubation at 15 °C generally caused a slight reduction in niche size from that at 25 °C. However, when only the 18 key wheat grain carbon sources were considered, the temperature effects on niche size were reversed. Species had a large impact on niche size. On average *F. poae* had the largest niche size of the fungi tested. This was regardless of environmental factors or whether 95 or the key 18 carbon sources were considered. The smallest average overall niche sizes was for *F. graminearum* when considering 95 carbon sources, and *M. nivale* var. *majus* when only considering the key 18 carbon sources. The impact of environmental factors on niche sizes was not uniform between species. For instance, changes in niche sizes for *F. culmorum* due to  $a_w$  levels were small compared to *F. graminearum* and *M. nivale* var. *majus* which were subject to larger reductions in niche size when  $a_w$  was lowered to 0.93  $a_w$ .



### 3.6.1 Niche overlap and niche maps

The NOI scores in Table 3.26 represent the following;  $\text{NOI}_{(\text{F.c})}$  is the percentage of carbon sources used by *F. culmorum* that are also used by the other species, and  $\text{NOI}_{(\text{spp})}$  is the percentage of carbon sources that an organism can metabolise that can also be metabolised by *F. culmorum*. A score of >90% (expressed as 0.90 in the table) indicates that the fungus in question has the same ecological niche as the target organism. When using the 95 carbon source assay  $\text{NOI}_{(\text{spp})}$  indicates that all the species occupy the same niche as *F. culmorum* at 25 °C and 0.93  $a_w$ . However, at 0.995  $a_w$  the  $\text{NOI}_{(\text{F.c})}$  indicates that only *F. graminearum*, *F. poae* and *M. nivale var. majus* shared the *F. culmorum* niche. When only considering the 18 key carbon sources, NOI results indicate that most of the fungi occupy the same niche as *F. culmorum* regardless of the temperature or  $a_w$  levels.

NOI result are one dimensional and do not singularly show how similar a particular fungus's niche is related to the niche of the target fungus, thus the need to calculate both  $\text{NOI}_{(\text{F.c})}$  and  $\text{NOI}_{(\text{spp})}$ . By plotting  $\text{NOI}_{(\text{F.c})}$  against  $\text{NOI}_{(\text{spp})}$  to form a niche map it is easy to see the relationship between each fungus and the target organism's niche. Figures 3.41 to 3.44 show the niche maps for both the 95 and 18 carbon source NOI results. Each niche map has four distinct regions;-

1.  $\text{NOI}_{(\text{F.c})}$  and  $\text{NOI}_{(\text{spp})} = >0.9$  Target niche region, occupied by coexisting species, obligate niche organisms:- they must occupy the same niche as the target pathogen.
2.  $\text{NOI}_{(\text{F.c})}$  and  $\text{NOI}_{(\text{spp})} \leq 0.9$  Separate niche occupation to the target pathogen.
3.  $\text{NOI}_{(\text{F.c})} = >0.9$   $\text{NOI}_{(\text{spp})} \leq 0.9$  coexistence species, strict obligate niche organisms:- not only can these organisms only exists within the niche of the target pathogen but they can metabolise fewer carbon sources within this niche than the target.
4.  $\text{NOI}_{(\text{F.c})} \leq 0.9$   $\text{NOI}_{(\text{spp})} = >0.9$  coexisting species, facultative niche organisms:- able to utilize some (>90%) of the same carbon sources as the target organism but not restricted to the target pathogen's niche.

In addition to the region of the map a fungus falls into, the further it is from the target niche region the more dissimilar is its niche to that of the target organism. Figure 3.41 shows the niche maps for 95 carbon sources assays at 0.995  $a_w$ , at 15 and 25 °C. This identifies *A. tenuissima*, *C. herbarum* and *M. nivale* var. *majus* as occupying separate niches to *F. culmorum* at both 15 and 25 °C. *F. graminearum*, *F. poae* and *P. verrucosum* niches under these conditions are strict obligates of *F. culmorum*'s niche. At 0.93  $a_w$  and 25 °C (Figure 3.42) all the fungi were able to metabolise the *F. culmorum* multnutritional range and additional carbon sources outside the niche. When the temperature was adjusted to 15 °C, *F. poae*'s niche changed to the same as that of *F. culmorum*, and *F. graminearum* now occupied a separate niche.

Niche maps constructed using only the 18 key wheat carbon sources are shown in Figures 3.43 and 3.44. At 0.995  $a_w$  (Figure 3.43) and 0.93  $a_w$  (Figure 3.44) all of the fungi can exist in the target niche. At 0.995  $a_w$  and 25 °C *C. herbarum* and *M. nivale* var. *majus* were also able to metabolise carbon sources outside the *F. culmorum* niche. *F. graminearum*, *F. poae*, *M. nivale* var. *majus* and *P. verrucosum* are strict obligates of the *F. culmorum* niche at 0.995  $a_w$  and 15 °C. At 0.93  $a_w$  and 25 °C *A. tenuissima*, *C. herbarum*, *F. graminearum* and *P. verrucosum* were facultative niche organisms. *F. poae* occupied the same niche as *F. culmorum*, and *M. nivale* var. *majus* occupied a separate niche. When incubated at 15 °C, *M. nivale* var. *majus* became a facultative niche organism and *F. graminearum* moved to a niche separate from *F. culmorum*'s.



**Table 3.26** Niche size and niche index results determined from carbon source utilization assays encompassing 95 (A and B) or 18 (C and D) carbon sources. Assays were carried out at two different  $a_w$  levels and incubated at 15 °C (A and C) or 25 °C (B and D). Target niche was that of *F. culmorum*.

A.

	0.93 $a_w$			0.995 $a_w$		
	Niche Size	NOI <sub>(F.c)</sub>	NOI <sub>(Spp)</sub>	Niche Size	NOI <sub>(F.c)</sub>	NOI <sub>(Spp)</sub>
<i>F. culmorum</i>	18			16		
<i>A. tenuissima</i>	3	0.17	1	17	1	0.94
<i>C. herbarum</i>	14	0.78	1	17	1	0.94
<i>F. graminearum</i>	0	0	0	17	0.94	0.88
<i>F. poae</i>	17	0.94	1	18	1	0.89
<i>M. majus</i>	2	0.11	1	18	1	0.89
<i>P. verrucosum</i>	17	0.94	1	18	1	0.89

B.

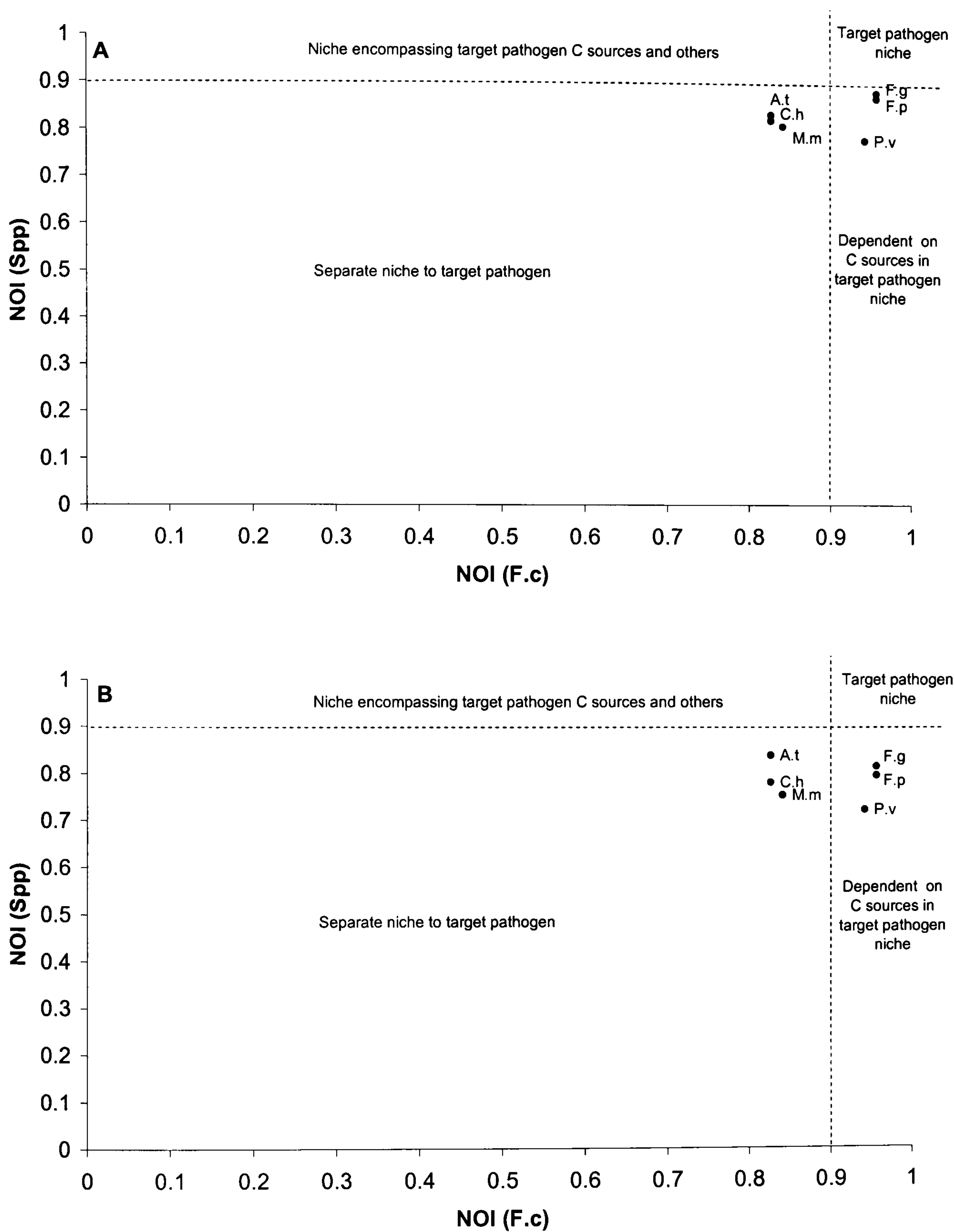
	0.93 $a_w$			0.995 $a_w$		
	Niche Size	NOI <sub>(F.c)</sub>	NOI <sub>(Spp)</sub>	Niche Size	NOI <sub>(F.c)</sub>	NOI <sub>(Spp)</sub>
<i>F. culmorum</i>	18	0	0	17	0	0
<i>A. tenuissima</i>	7	0.39	1	17	0.94	0.94
<i>C. herbarum</i>	11	0.61	1	16	0.88	0.94
<i>F. graminearum</i>	7	0.39	1	18	1	0.94
<i>F. poae</i>	18	1	1	18	1	0.94
<i>M. majus</i>	0	0	0	16	0.88	0.94
<i>P. verrucosum</i>	15	0.83	1	18	1	0.94

C.

	0.93 $a_w$			0.995 $a_w$		
	Niche Size	NOI <sub>(F.c)</sub>	NOI <sub>(Spp)</sub>	Niche Size	NOI <sub>(F.c)</sub>	NOI <sub>(Spp)</sub>
<i>F. culmorum</i>	93			69		
<i>A. tenuissima</i>	27	0.29	1	68	0.83	0.84
<i>C. herbarum</i>	49	0.53	1	73	0.83	0.78
<i>F. graminearum</i>	19	0.20	1	81	0.96	0.81
<i>F. poae</i>	80	0.86	1	83	0.96	0.80
<i>M. majus</i>	70	0.75	1	90	0.94	0.72
<i>P. verrucosum</i>	5	0.05	1	77	0.84	0.75

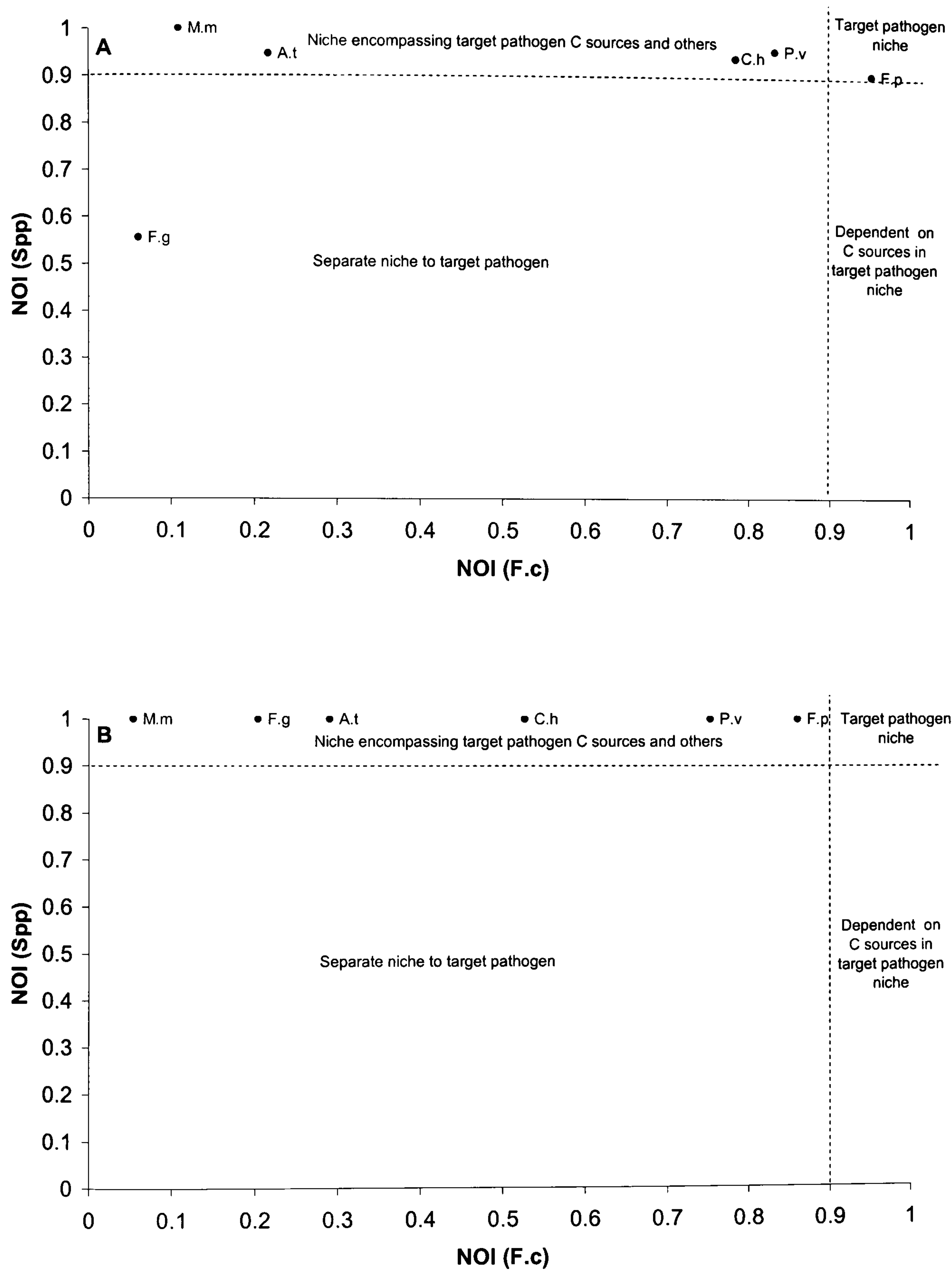
D.

	0.93 $a_w$			0.995 $a_w$		
	Niche Size	NOI <sub>(F.c)</sub>	NOI <sub>(Spp)</sub>	Niche Size	NOI <sub>(F.c)</sub>	NOI <sub>(Spp)</sub>
<i>F. culmorum</i>	83			73		
<i>A. tenuissima</i>	19	0.22	0.95	78	0.83	0.83
<i>C. herbarum</i>	69	0.78	0.94	84	0.83	0.82
<i>F. graminearum</i>	9	0.06	0.56	76	0.96	0.88
<i>F. poae</i>	87	0.95	0.91	77	0.96	0.87
<i>M. majus</i>	9	0.11	1	89	0.84	0.81
<i>P. verrucosum</i>	72	0.83	0.96	91	0.94	0.78

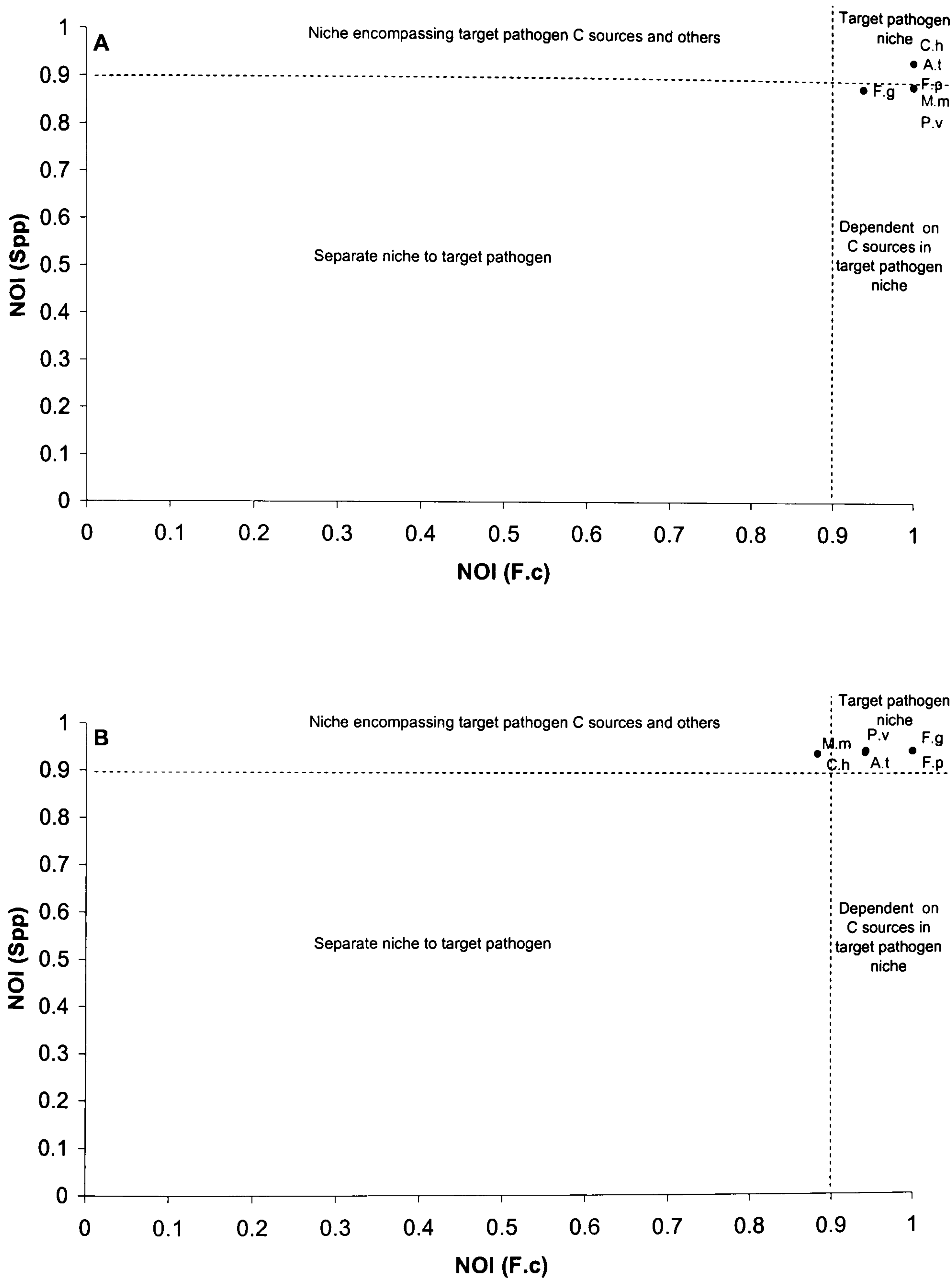


**Figure 3.41** Niche map encompassing 95 carbon sources, target organism *F. culmorum* (UK3). Water activity was adjusted to 0.995  $a_w$  and incubation was at:- A, 15 °C B, 25 °C. Species key, F.g *F. graminearum*, F.p *F. poae*, M.m *M. nivale* var. *majus*, A.t *A. tenuissima*, C.h *C. herbarum* and P.v *P. verrucosum*.



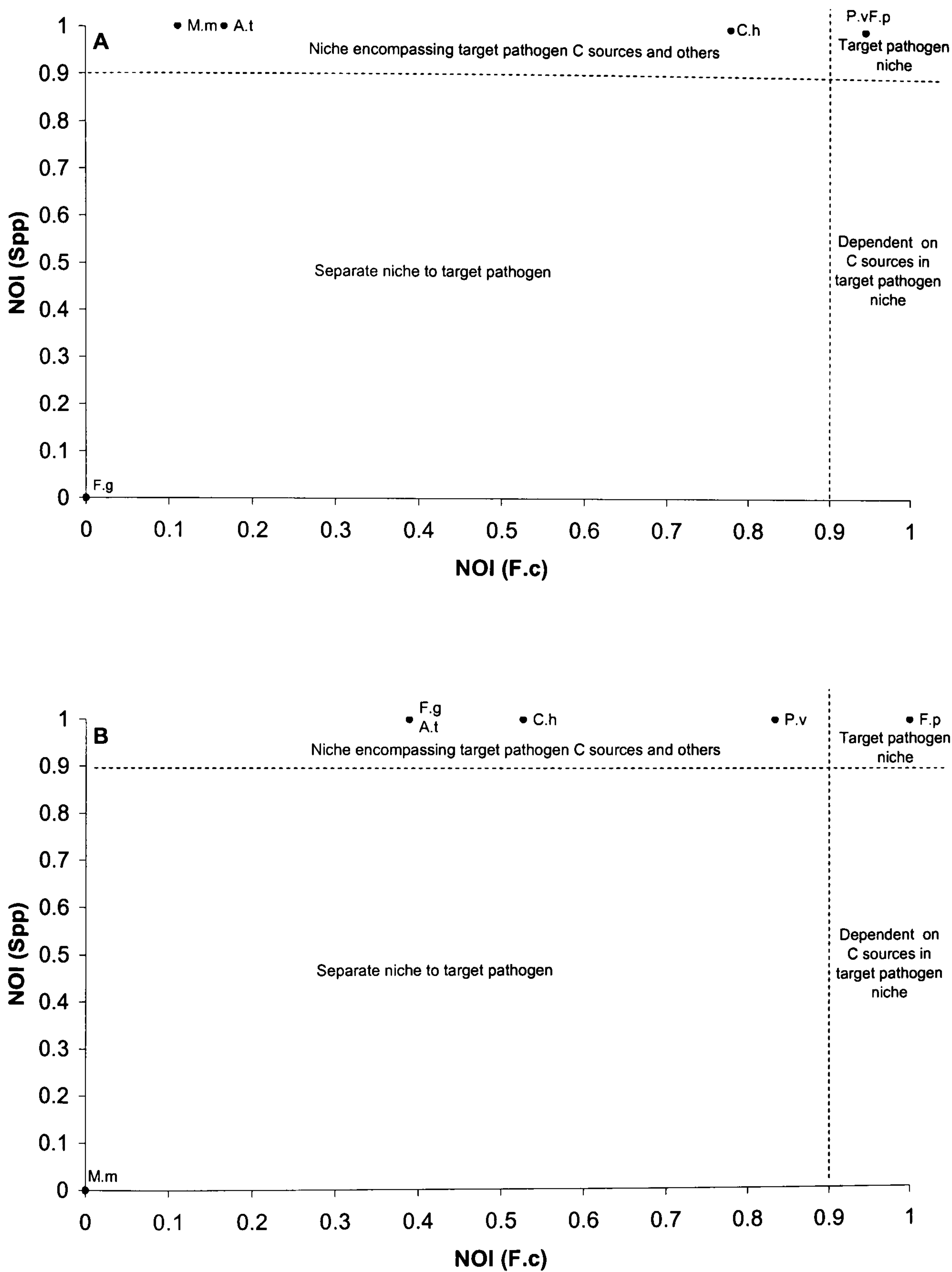


**Figure 3.42** Niche map encompassing 95 carbon sources, target organism *F. culmorum* (UK3). Water activity was adjusted to 0.930  $a_w$  and incubation was at:- A, 15 °C B, 25 °C. Species key, F.g *F. graminearum*, F.p *F. poae*, M.m *M. nivale* var. *majus*, A.t *A. tenuissima*, C.h *C. herbarum* and P.v *P. verrucosum*.



**Figure 3.43** Niche map encompassing 18 carbon sources, target organism *F. culmorum* (UK3). Water activity was adjusted to 0.995  $a_w$  and incubation was at:- A, 15 °C B, 25 °C. Species key, F.g *F. graminearum*, F.p *F. poae*, M.m *M. nivale* var. *majus*, A.t *A. tenuissima*, C.h *C. herbarum* and P.v *P. verrucosum*.





**Figure 3.44** Niche map encompassing 18 carbon sources, target organism *F. culmorum* (UK3). Water activity was adjusted to 0.930  $a_w$  and incubation was at:- A, 15 °C B, 25 °C. Species key, F.g *F. graminearum*, F.p *F. poae*, M.m *M. nivale* var. *majus*, A.t *A. tenuissima*, C.h *C. herbarum* and P.v *P. verrucosum*.

### **3.7 HYDROLYTIC ENZYME PRODUCTION BY WHEAT GRAIN NICHE FUNGI ON IRRADIATED WHEAT GRAIN UNDER VARIOUS $a_w$ AND TEMPERATURE CONDITIONS**

The carbon source utilization assays (see previous section) identified the niches of some common wheat grain fungi and the relationship of these niches with that of *F. culmorum*. However, it does not fully explain why one fungus may be more dominant in a particular niche than another species. This experiment studied the impact of  $a_w$  and temperature on the quantitative hydrolytic enzymes produced by the fungi studied in sections 3.4 to 3.6 . Previous studies have shown that hydrolytic enzymes can play an important role in the colonisation and growth of a fungus on a nutritional substrate (Marin *et al.*, 1997b; Marin *et al.*, 1998b). The enzyme activity profiles generated by this experiment in combination with the findings of the interaction and carbon source utilization experiments form part of a comprehensive study to understand how and why *F. culmorum* and similar species compete with in the grain ecosystem. When this information is combined with growth and mycotoxin profiles for *F. culmorum* and *F. graminearum* (see Section 3.2 ) it greatly improves upon the information that has previously been available for determining critical control points within the wheat grain ecosystem..

#### **3.7.1 Levels of total and specific enzyme activity produced by different wheat grain niche fungi**

Enzyme activity is quantified in this experiment as total activity and specific activity. Total activity is the overall increase or decrease in activity. An increase in total activity can be caused by the following: an increase in the amount of enzyme present, an increase of the activity of enzymes present or a combination of both factors. Specific activity is the level of activity relative to the amount of protein present. These two measurements of enzyme activity complement each other reflecting enzyme dynamics and behaviour expressed by a particular fungus during colonization of a substrate, in this case on irradiated wheat grain adjusted to various  $a_w$  levels.



Figures showing the temporal total and specific activity results obtained for the seven enzymes produced by the different spoilage fungi across the various environmental conditions tested, are located in the appendix (Appendix I,I.I ). The hydrolytic enzymes  $\alpha$ -D-galactosidase,  $\beta$ -D-glucosidase,  $\beta$ -D-xylosidase and *N*-acetyl- $\beta$ -D-glucosaminidase were produced readily by all of the fungi.  $\beta$ -D-fucosidase,  $\alpha$ -D-mannosidase and *N*-acetyl- $\alpha$ -D-glucosaminidase activity was generally not present or low. The exceptions to this were total activity of  $\beta$ -D-fucosidase by *A. tenuissima* and total activity of  $\alpha$ -D-mannosidase by *F. culmorum*, *F. graminearum* and *P. verrucosum*. Figure 3.45 shows an example of total and specific activity for seven different enzymes produced by one of the key species tested (*F. culmorum*) on irradiated wheat grain incubated for 14 days at 0.99  $a_w$  and 25°C.

Figure 3.46 Compares total and specific activities of an enzyme, in this case  $\beta$ -D-glucosidase. The highest average total activity was for  $\beta$ -D-glucosidase produced by *P. verrucosum*.  $\beta$ -D-glucosidase was produced in the highest total activity of the seven enzymes in all of the fungi tested. However,  $\alpha$ -D-galactosidase almost achieved the same total activity as  $\beta$ -D-glucosidase in the *F. culmorum* and *M. nivale var. majus* samples. Specific activities for  $\beta$ -D-glucosidase were on average higher than any of the other enzymes for all of the fungi except *M. nivale var. majus*, where  $\alpha$ -D-galactosidase activity was higher. A particular point of interest is that the *Fusaria* have a higher *N*-acetyl- $\beta$ -D-glucosaminidase average specific activity than any of the other fungi.

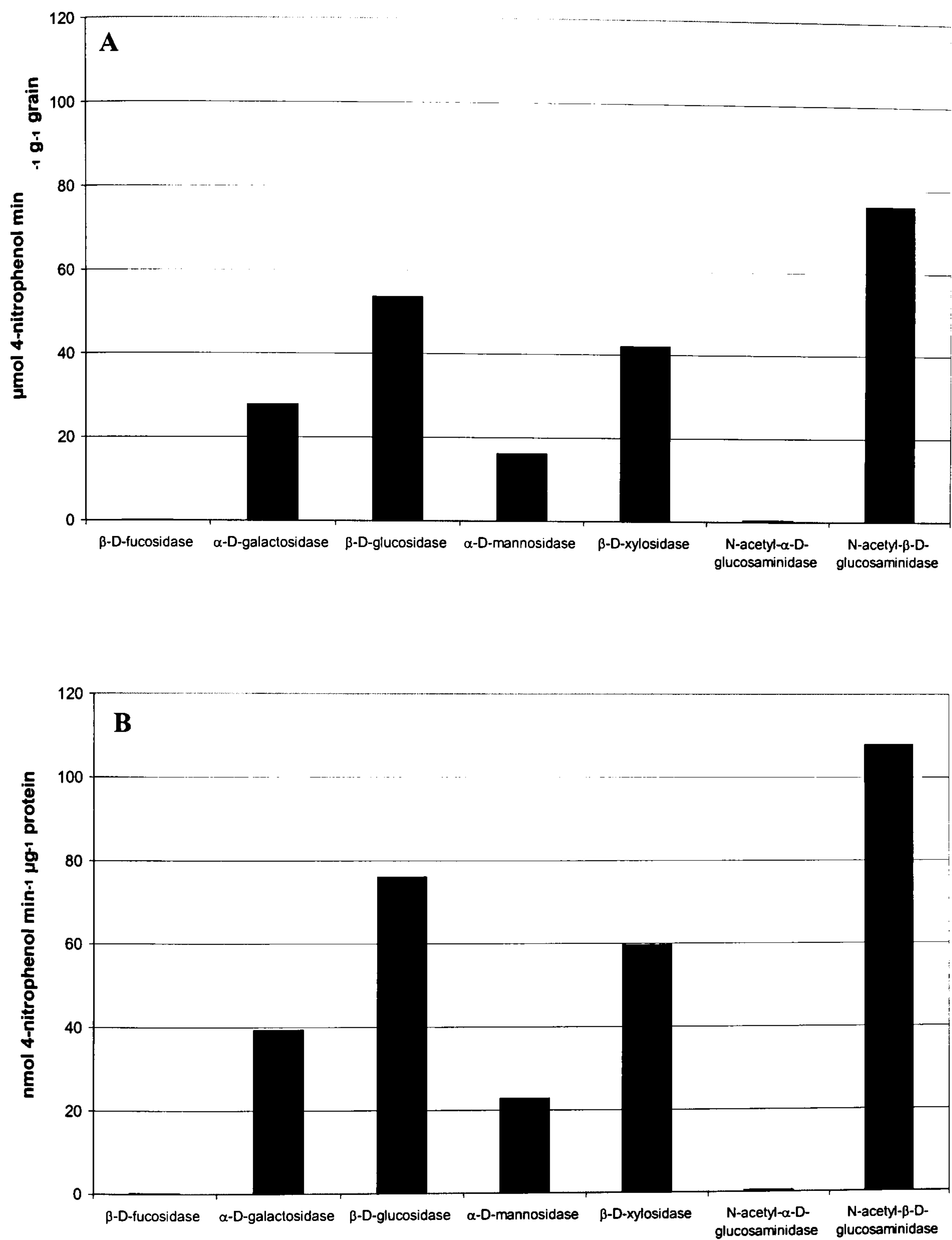
### 3.7.2 Effect of abiotic factors on total and specific enzyme activities

Temperature,  $a_w$ , level and time had an effect on both the total and specific enzyme activities. Temperature generally caused a small increase in both total and specific activities when increased from 15 to 25 °C. Figure 3.47 shows an example of the effects of  $a_w$  and time on total and specific activity of  $\beta$ -D-glucosidase produced by *F. culmorum* at 25°C. Longer incubation periods also resulted in an increase in the enzyme activities. However, this was markedly greater for total than specific activity.

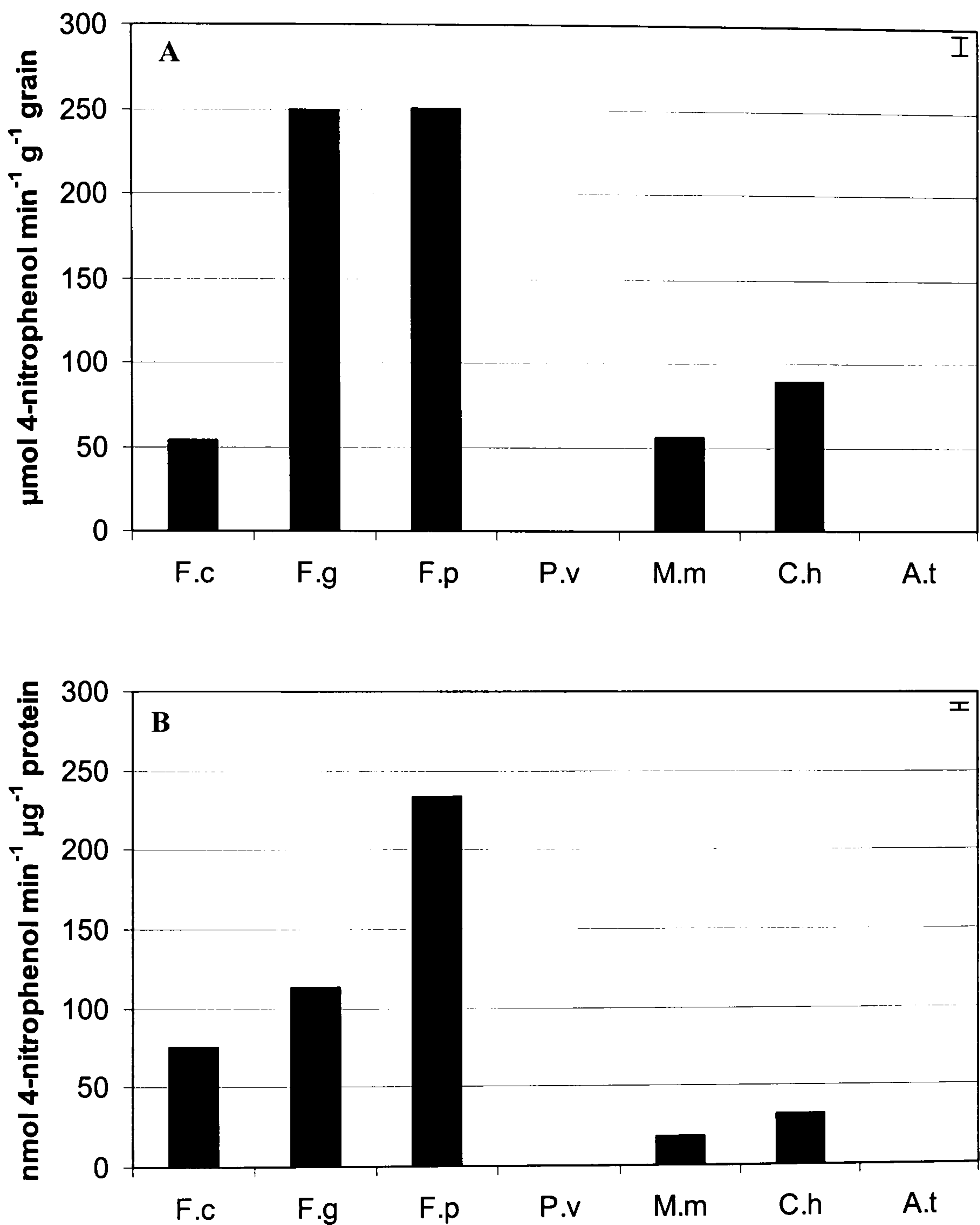
The  $a_w$  level of the irradiated wheat grain had a large effect on total and specific of enzyme activity. Generally, enzyme activity was seen to increase as  $a_w$  was increased.

ANOVA (See appendix I Section I.II Table I.9) analysis of total activity indicates that all the experimental factors and their interactions were significant ( $P < 0.01$ ). Analysis of specific activity by ANOVA (See appendix I Section I.II Table I.9) showed that all the factors and their interactions, apart from temperature as a single factor were significant. This was the case for all species tested.



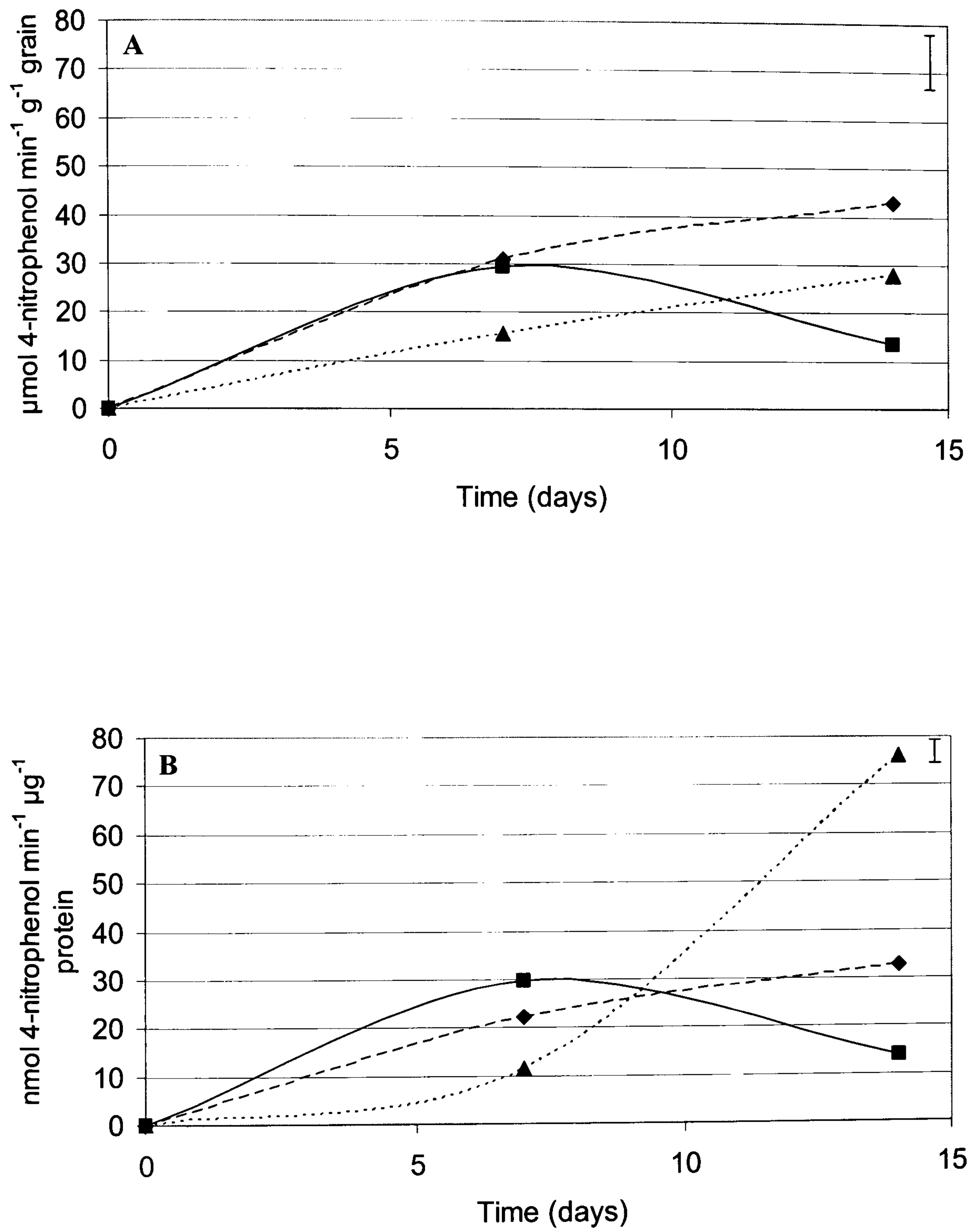


**Figure 3.45** Example of enzyme total (A) and specific (B) activity for seven different enzymes produced by *F. culmorum* on irradiated wheat grain incubated for 14 days at 0.99  $a_w$  and 25°C.



**Figure 3.46** Example of the interspecies difference between total (A) and specific (B) activities of  $\beta$ -D-glucosidase at 0.99  $a_w$  25°C after 14 days incubation on irradiated wheat grain. Species key:- F.c, *F. culmorum* F.g *F. graminearum*, F.p *F. poae*, M.m *M. nivale* var. *majus*, A.t *A. tenuissima*, C.h, *C. herbarum* and P.v *P. verrucosum*. Bar represents LSD.





**Figure 3.47** Examples of the effects of time and  $a_w$  (▲ 0.99  $a_w$ , ■ 0.95  $a_w$  and ◆ 0.93  $a_w$ ) on total (A) and specific (B) activity of  $\beta$ -D-glucosidase by *F. culmorum* on irradiated wheat grain at 25°C. Bar represents LSD

### 3.8 ESSENTIAL OILS AND ANTIOXIDANTS AS CONTROLS FOR *FUSARIUM* GROWTH AND TOXIN PRODUCTION STUDIES

Essential oils and antioxidants were screened for antifungal properties. The five most effective essential oils and antioxidants from this screening process were then studied further in detailed *in vitro* (on wheat agar) and *in situ* (irradiated wheat grain) studies. The detailed studies determined the effect of the oils on growth of three different *Fusarium* species and trichothecene production across a range of temperatures and  $a_w$  levels.

#### 3.8.1 Initial screen

The initial screen of the essential oils was a zone of inhibition assay. Of the 23 essential oils tested only three of them (Bay, Cinnamon and Clove) were able to produce zones of inhibition. Figure 3.48 shows the zones of inhibition produced by each of these oils in the assay. The methanol control did not produce any zones of inhibition in any of the samples. The initial screen of the six antioxidants was a temporal growth study (Figure 3.49). BHA and propyl paraben significantly ( $P > 0.05$ ) inhibited growth. BHT did exhibit fungicidal properties, but these were not significant. Based on the findings of this screen Bay, BHA, Cinnamon, Clove and Propyl paraben were studied further.

#### 3.8.2 Detailed *in vitro* study on the efficacy of essential oils and antioxidants on *Fusarium* growth

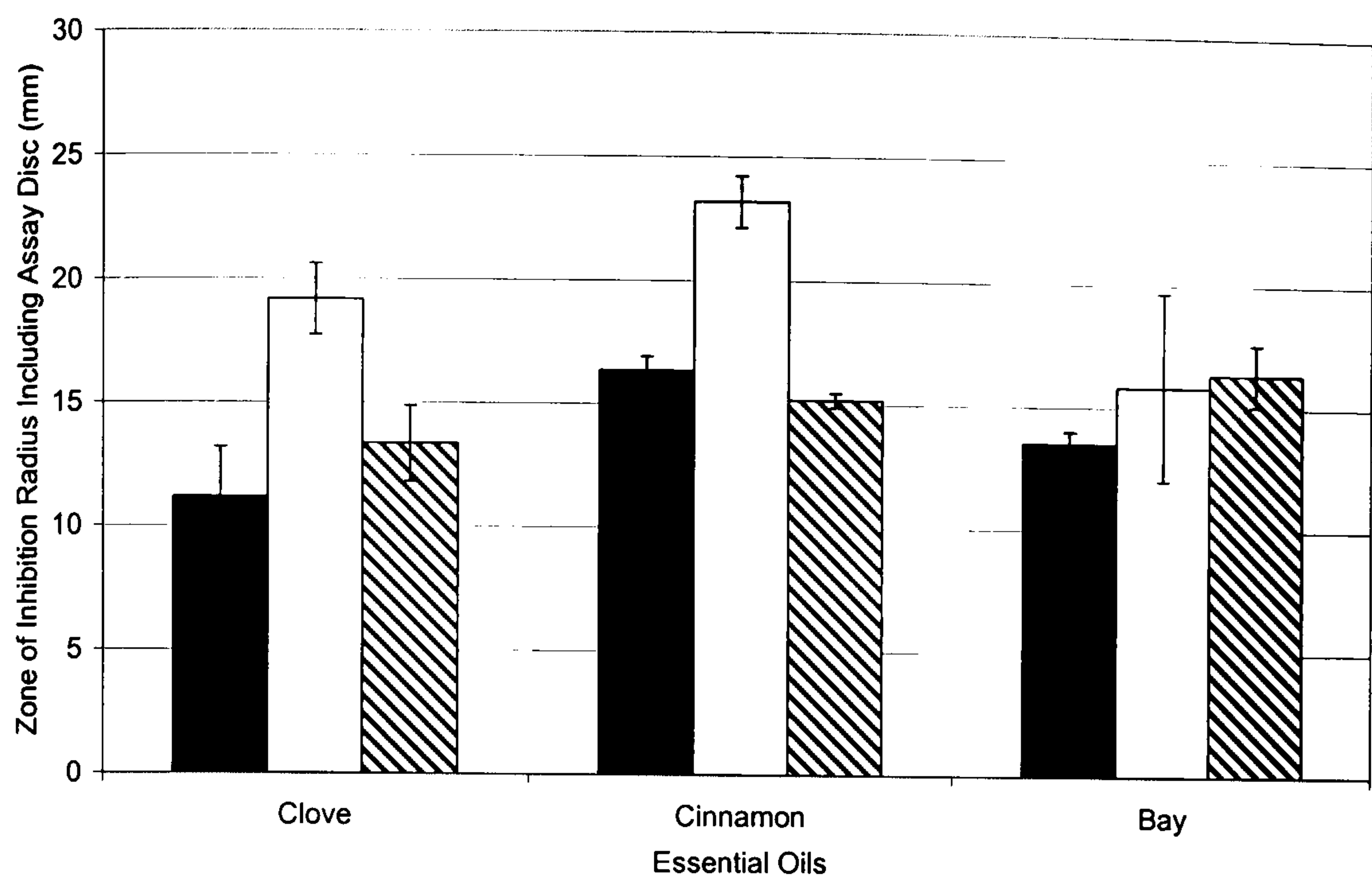
Figures 3.56-3.58 show the effect of five essential oils or antioxidants, at different concentrations and under various environmental conditions on growth of *F. culmorum*, *F. graminearum* and *F. poae* on wheat agar. Growth rates were affected by temperature,  $a_w$ , essential oil or antioxidant present and its concentration. Generally, growth was inhibited at lowered  $a_w$  levels. However, stimulation of *F. poae* was seen at 0.982  $a_w$ .



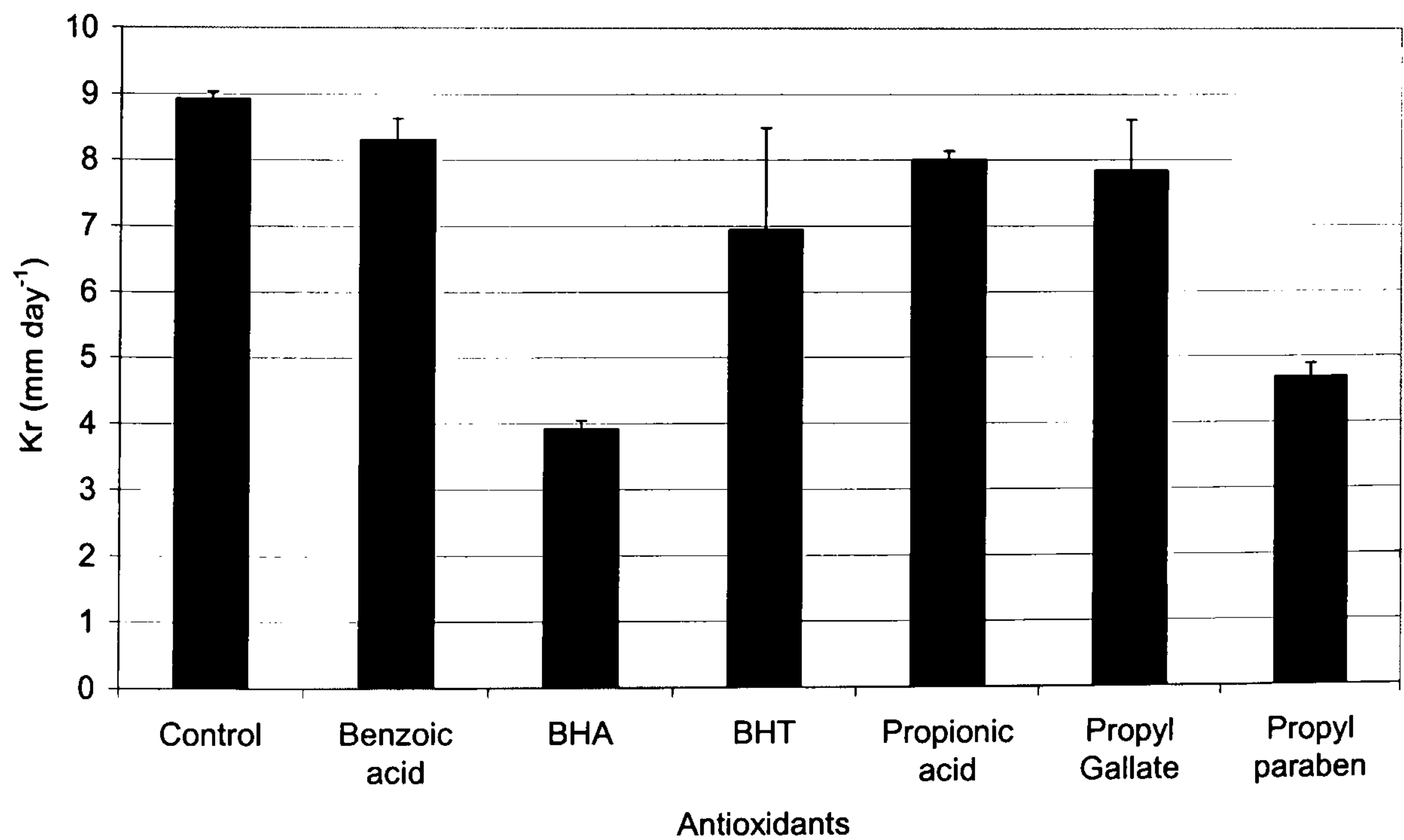
There was no growth on any plates that contained  $\geq 500$  ppm of essential oil or antioxidant. There was significant inhibition of growth at 100 ppm concentrations for most of the control agents. Inhibition of growth was not uniform across all isolates. For example, bay was not as effective against *F. culmorum* and *F. graminearum* as against *F. poae*. Table 3.27 shows the LD<sub>50</sub> values for the essential oils and antioxidants tested. LD<sub>50</sub> values were lower at the lower  $a_w$  and temperature tested. Low concentrations of essential oils and antioxidants stimulated growth of the isolates. However, this stimulation did not always occur at the same environmental conditions. For instance, low concentrations of essential oil or antioxidant resulted in significant stimulation of growth of *F. poae* at 0.985  $a_w$ . This demonstrates the importance of including the  $a_w$  parameter in this study.

### 3.8.3 *In situ* study on the efficacy of essential oils and antioxidants on *Fusarium* growth and toxin production

Figures 3.56 to 3.61 display the mycelial extension rate (Kr) values obtained for *F. culmorum*, *F. graminearum* and *F. poea* from the *in situ* irradiated wheat grain experiments at two temperatures and under two  $a_w$  level. *F. culmorum* produced greater growth rates than *F. poae* and *F. graminearum* at 0.995  $a_w$  and 25 °C. At 0.955  $a_w$  25 °C *F. graminearum* and *F. poae* produced higher growth rates than *F. culmorum*. All of the fungi tested produced dense aerial mycelium at high  $a_w$  and temperature. The effect of the different experimental variables, their levels and interactions on the Kr of the fungi is displayed graphically in Figure 3.62. The main effect plot clearly identifies cinnamon and clove essential oils as the most effective growth inhibiting agents. It is also clear that changes in treatment concentration and  $a_w$  elicited a large effect on growth rate. ANOVA (Table 3.28) tests showed that  $a_w$ , temperature, fungal species, treatment, and treatment concentration had a significant ( $P < 0.05$ ) effect on growth. Interactions between the experimental variables were also found to be of significance.

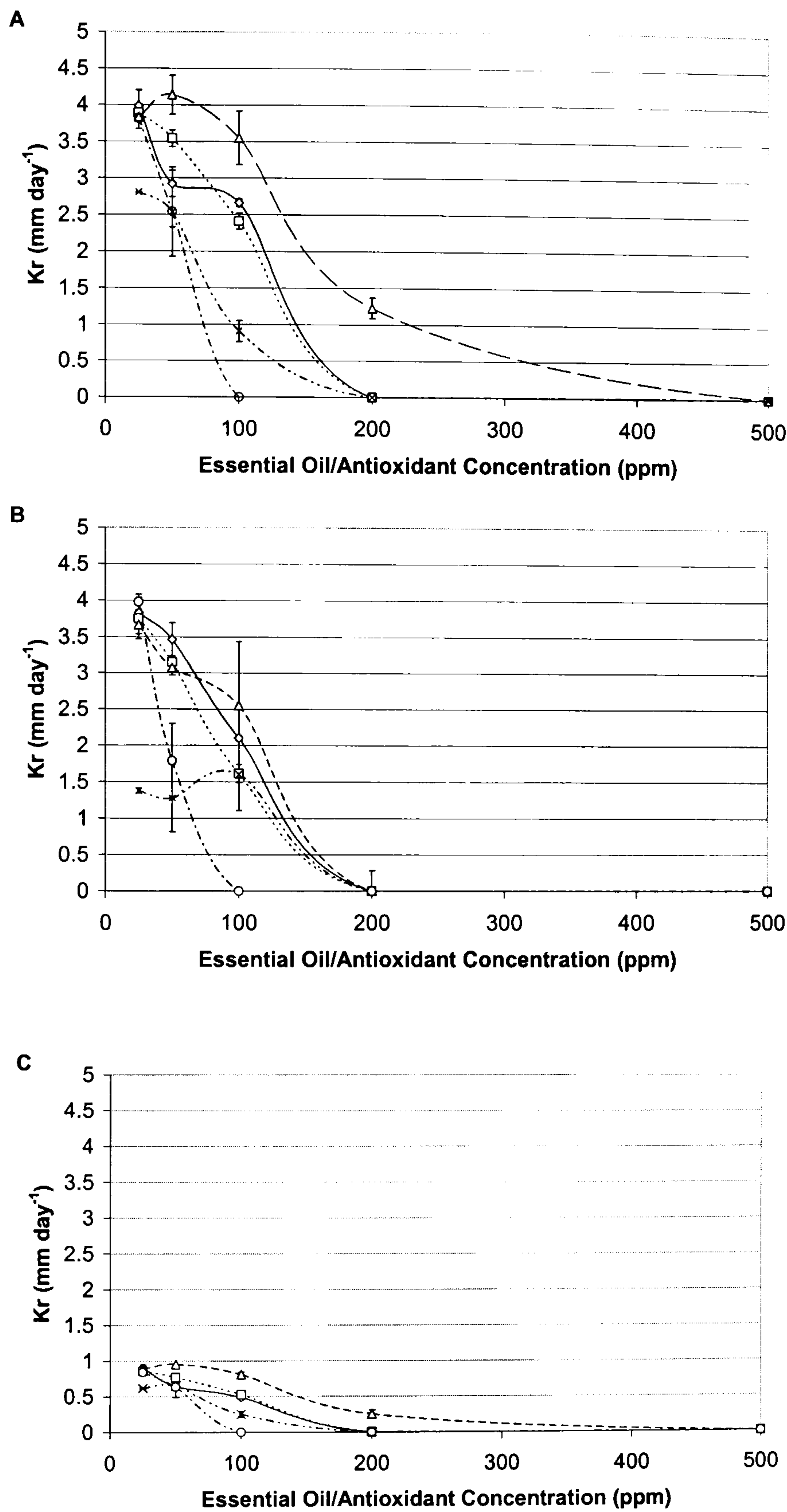


**Figure 3.48** Zones of inhibition formed in *F. culmorum* (strains:- ■ UK3, □ ITA1, ▨ NOR1) spread plates by 3 mm assay discs impregnated with 15  $\mu$ l of an essential oil (Dil: 1/10 methanol).

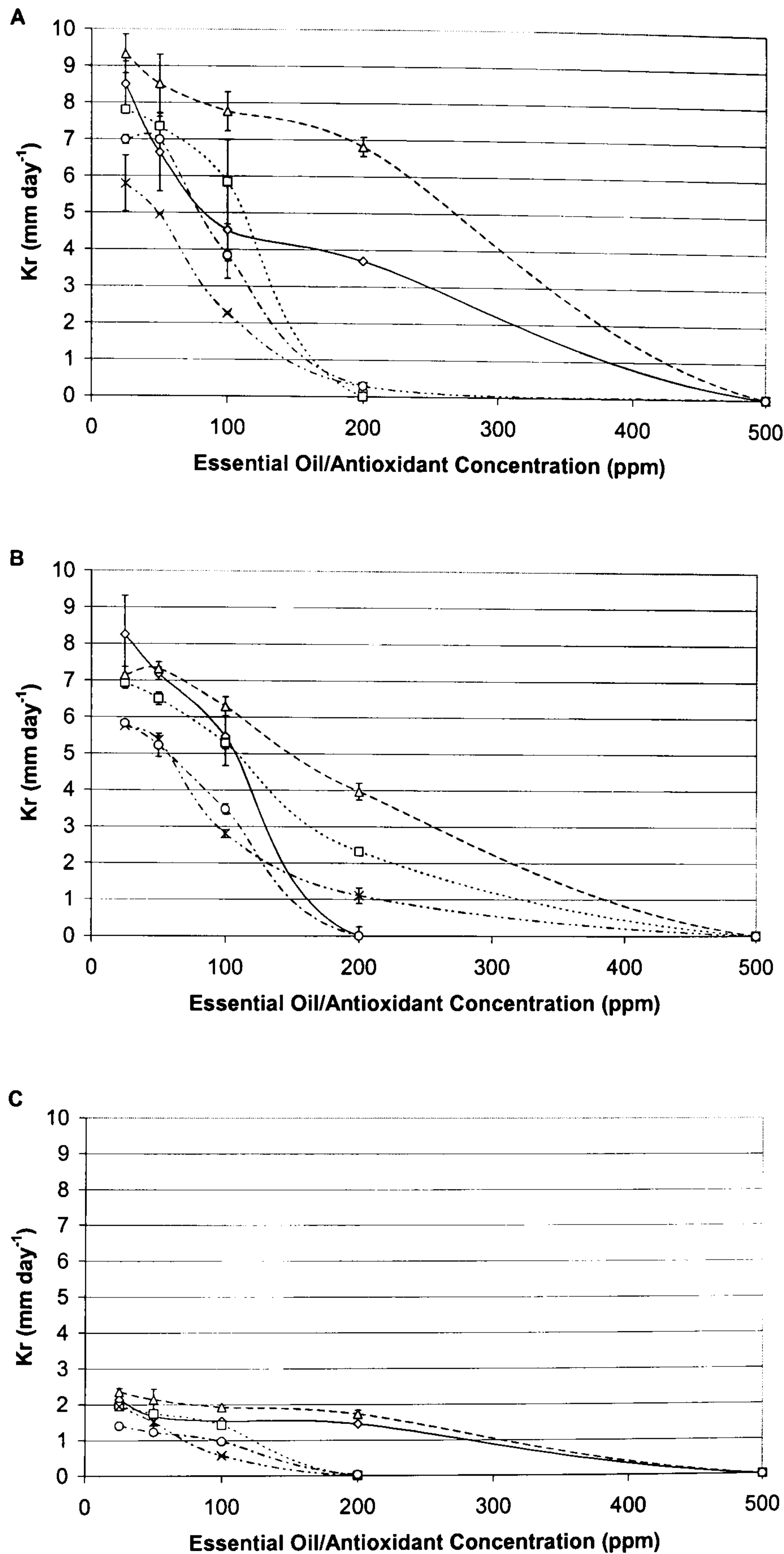


**Figure 3.49** Radial Growth of *F. culmorum* (UK3) on milled wheat agar, supplemented with 100 ppm of an antioxidant.



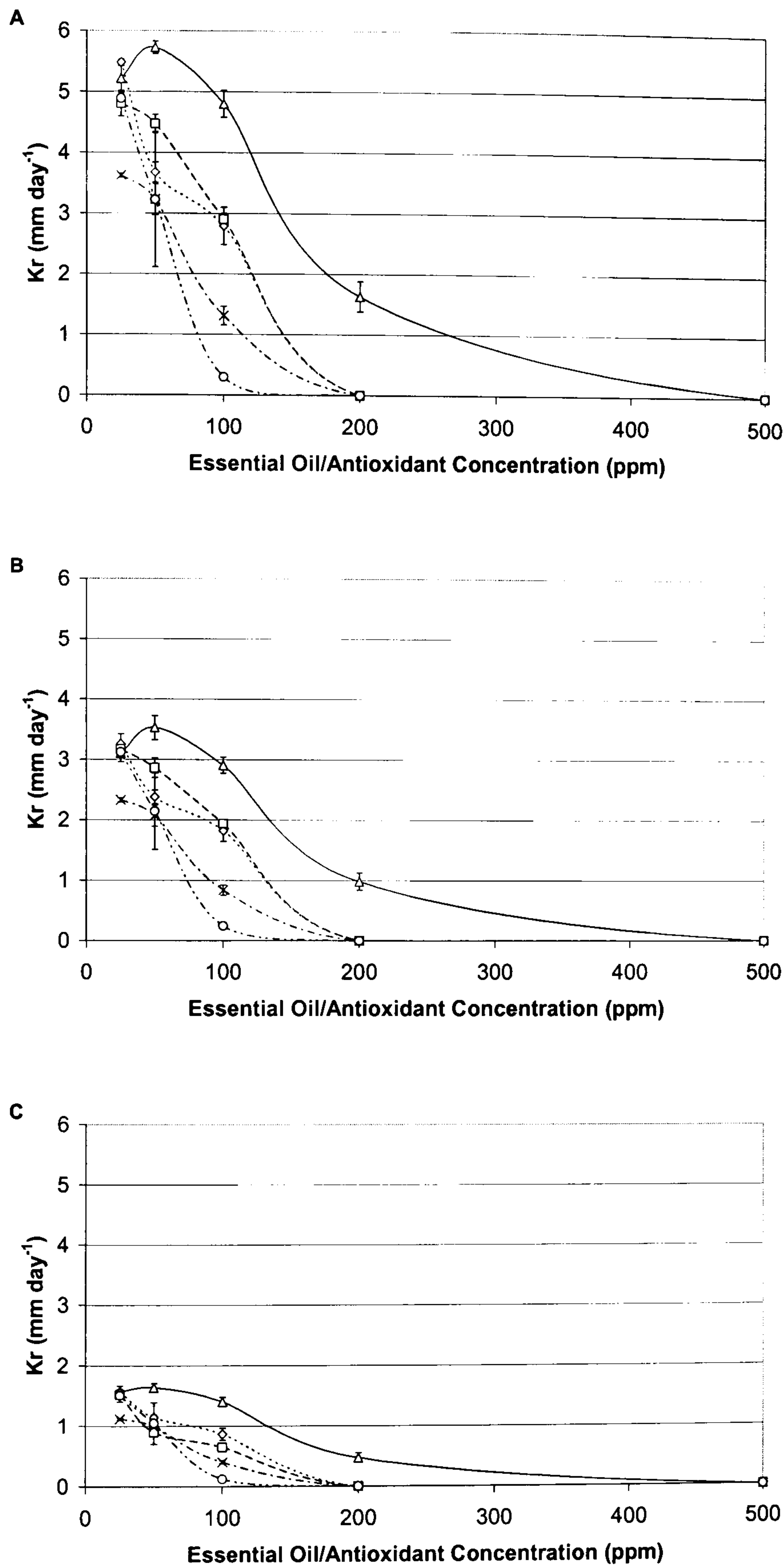


**Figure 3.50** Radial growth of *F. culmorum* (UK3) on milled wheat agar adjusted to A, 0.995  $a_w$ , B, 0.98  $a_w$ , and C, 0.955  $a_w$  supplemented with essential oil or antioxidant ( $\Delta$  bay, X BHA,  $\square$  cinnamon,  $\diamond$  clove,  $\circ$  propyl paraben) and incubated at 15 °C. Bars indicate standard deviation.

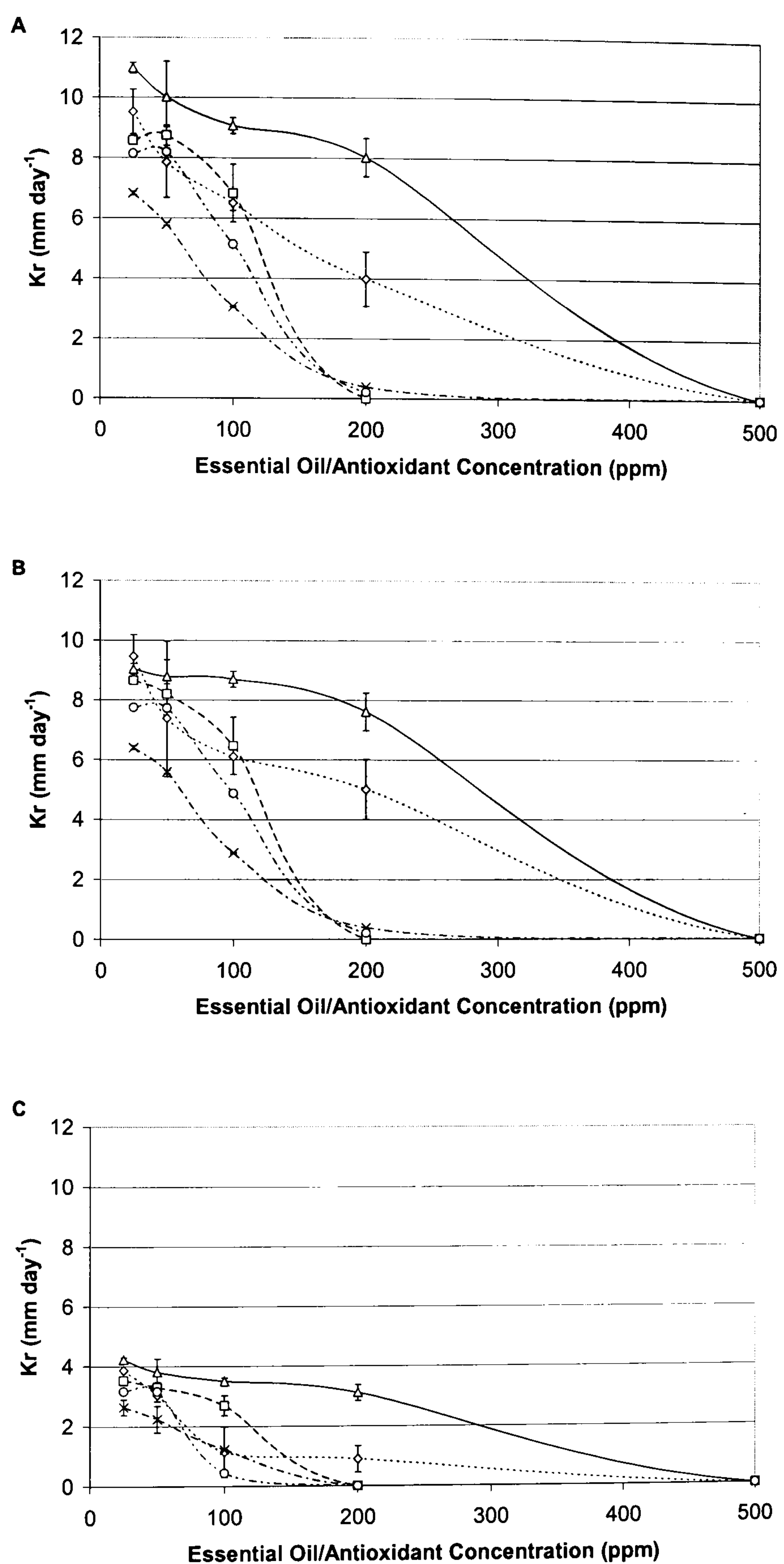


**Figure 3.51** Radial growth of *F. culmorum* (UK3) on milled wheat agar adjusted to A, 0.995  $a_w$ , B, 0.98  $a_w$ , and C, 0.955  $a_w$  supplemented with essential oil or antioxidant ( $\Delta$  bay, X BHA,  $\square$  cinnamon,  $\diamond$  clove,  $\circ$  propyl paraben) and incubated at 25 °C. Bars indicate standard deviation.



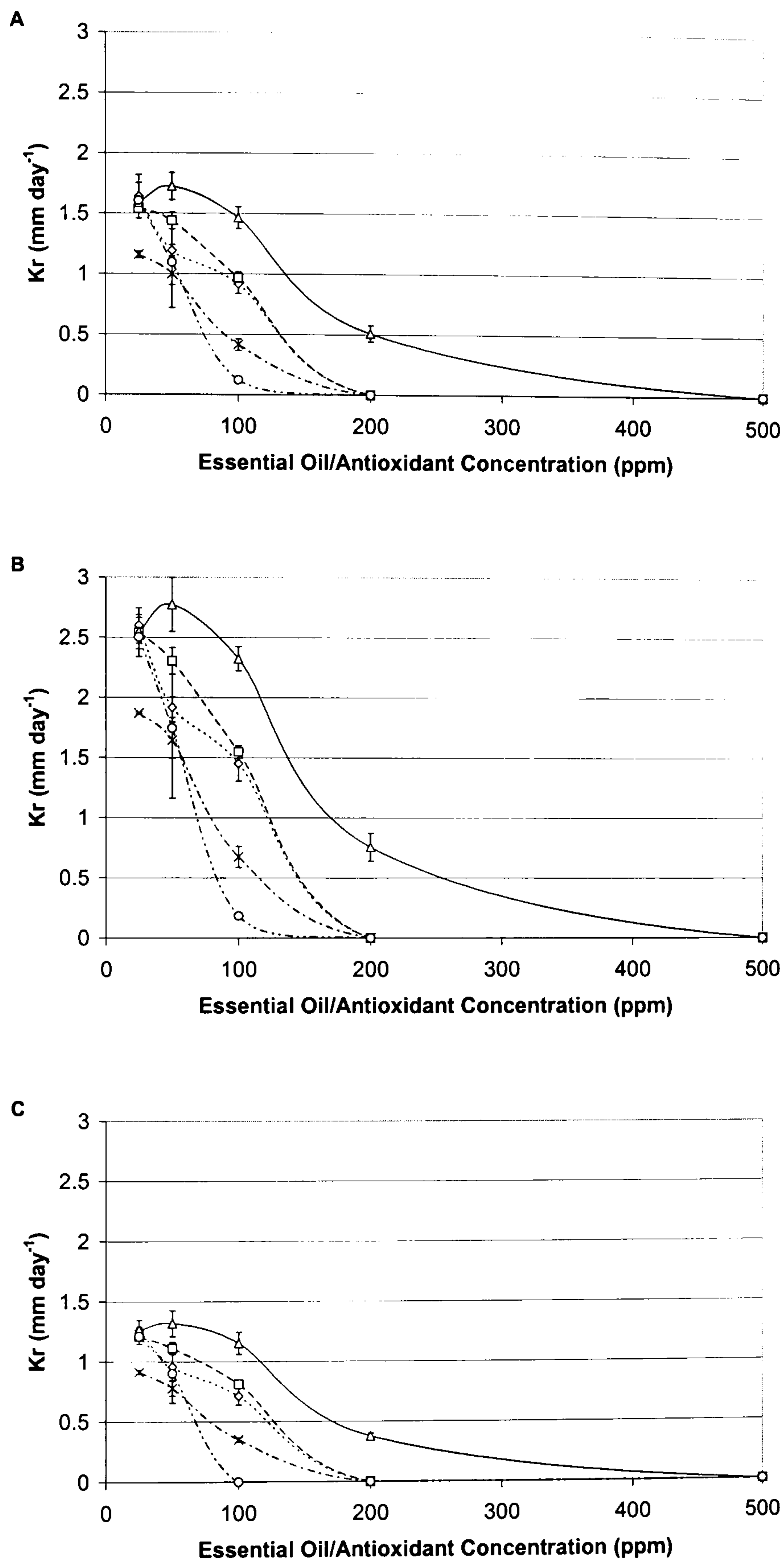


**Figure 3.52** Radial growth of *F. graminearum* on milled wheat agar adjusted to A, 0.995  $a_w$ , B, 0.98  $a_w$ , and C, 0.955  $a_w$  supplemented with essential oil or antioxidant ( $\Delta$  bay, X BHA,  $\square$  cinnamon,  $\diamond$  clove,  $\circ$  propyl paraben) and incubated at 15 °C. Bars indicate standard deviation.

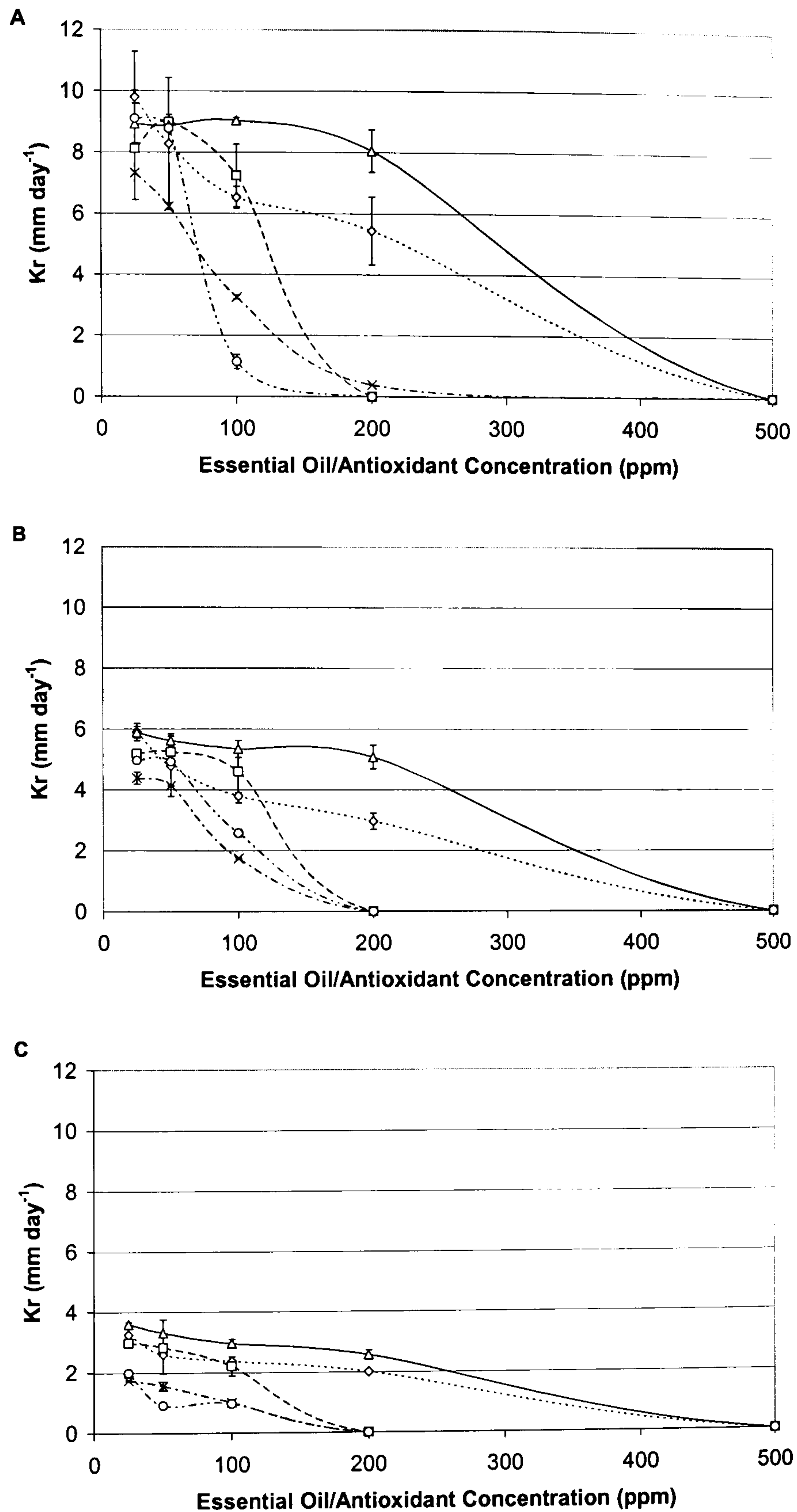


**Figure 3.53** Radial growth of *F. graminearum* on milled wheat agar adjusted to A, 0.995  $a_w$ , B, 0.98  $a_w$ , and C, 0.955  $a_w$  supplemented with essential oil or antioxidant ( $\Delta$  bay,  $\times$  BHA,  $\square$  cinnamon,  $\diamond$  clove,  $\circ$  propyl paraben) and incubated at 25 °C. Bars indicate standard deviation.





**Figure 3.54** Radial growth of *F. poae* on milled wheat agar adjusted to A,  $0.995 a_w$ , B,  $0.98 a_w$ , and C,  $0.955 a_w$  supplemented with essential oil or antioxidant ( $\Delta$  bay, X BHA,  $\square$  cinnamon,  $\diamond$  clove,  $\circ$  propyl paraben) and incubated at  $25^\circ\text{C}$ . Bars indicate standard deviation.



**Figure 3.55** Radial growth of *F. poae* on milled wheat agar adjusted to A, 0.995  $a_w$ , B, 0.98  $a_w$ , and C, 0.955  $a_w$  supplemented with essential oil or antioxidant ( $\Delta$  bay, X BHA,  $\square$  cinnamon,  $\diamond$  clove,  $\circ$  propyl paraben) and incubated at 25 °C. Bars indicate standard deviation.



**Table 3.27** LD<sub>50</sub> values of essential oils and antioxidants for control of growth of *F. culmorum*, *F. graminearum* and *F. poae*.

*F. culmorum*

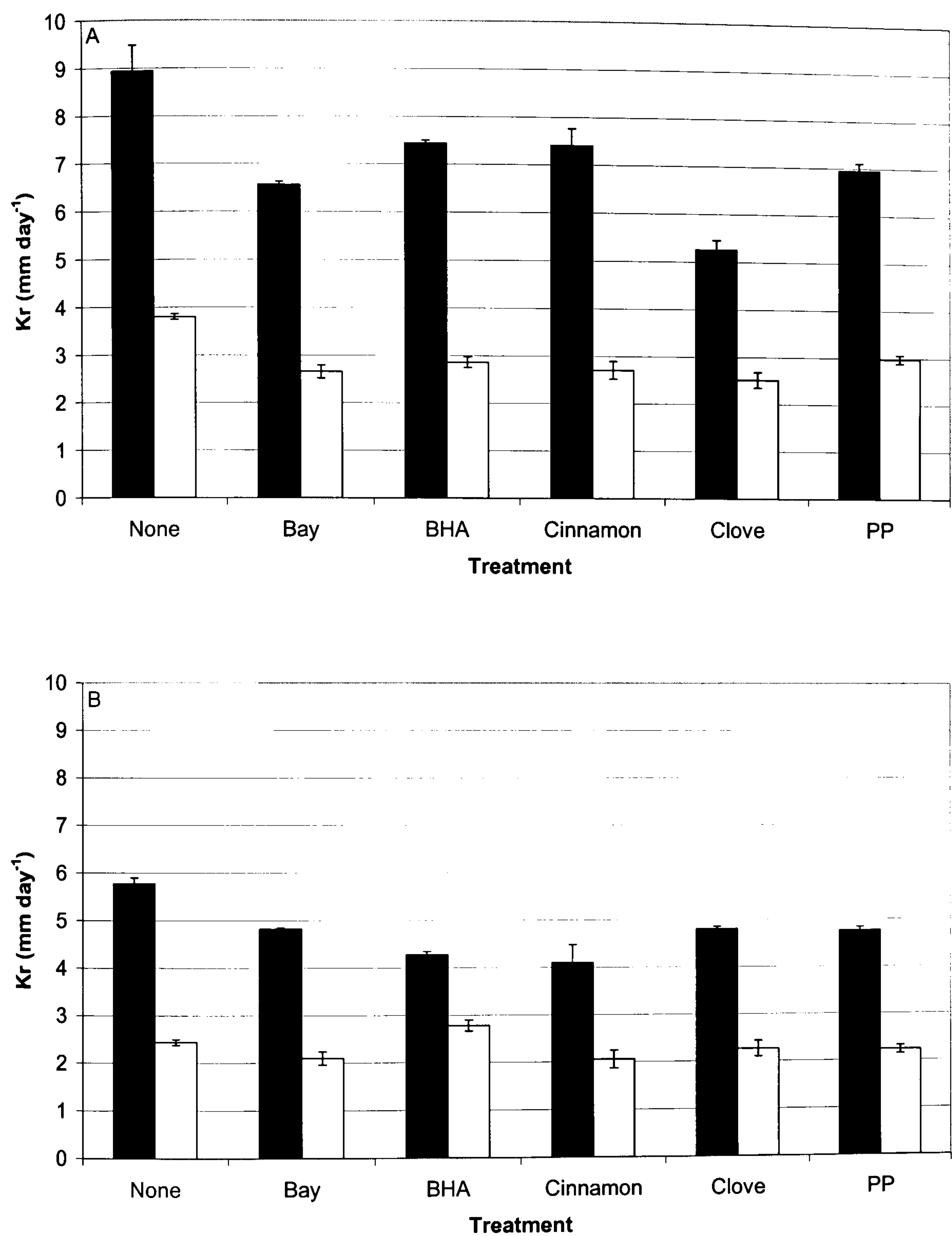
	15°C			25°C		
	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>
Bay	135	120	150	310	210	240
BHA	50	25	75	75	80	50
Cinnamon	105	90	105	130	140	100
Clove	110	110	105	190	120	190
Propyl paraben	75	50	100	100	90	25

*F. graminearum*

	15°C			25°C		
	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>
Bay	160	150	105	280	340	375
BHA	60	60	20	60	290	110
Cinnamon	110	110	40	115	130	150
Clove	110	105	40	140	95	110
Propyl paraben	60	55	40	100	125	75

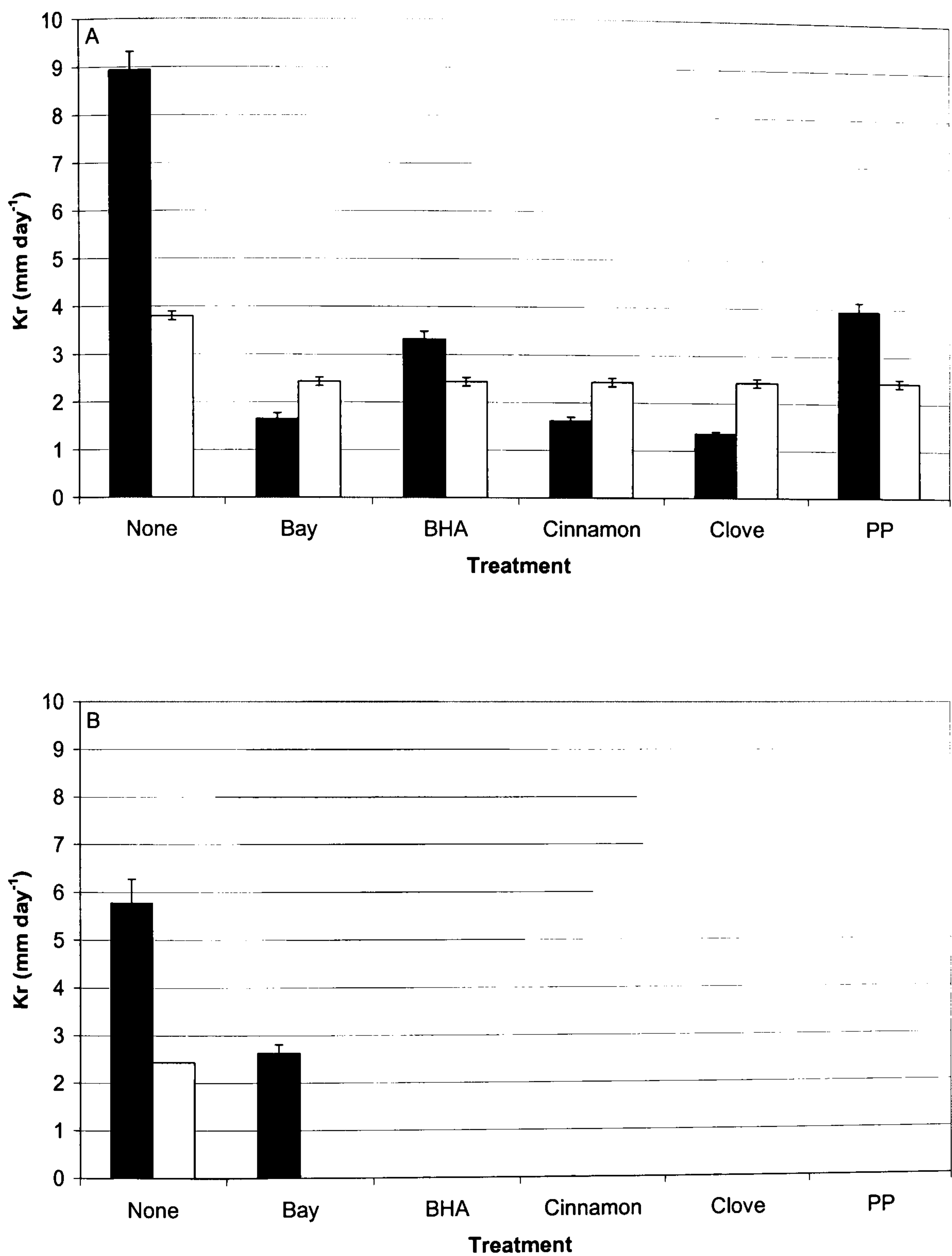
*F. poae*

	15°C			25°C		
	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>	0.995 a <sub>w</sub>	0.98 a <sub>w</sub>	0.955 a <sub>w</sub>
Bay	140	135	165	300	290	250
BHA	50	55	70	75	60	25
Cinnamon	85	105	120	120	115	105
Clove	100	95	110	220	175	200
Propyl paraben	50	55	65	75	85	25

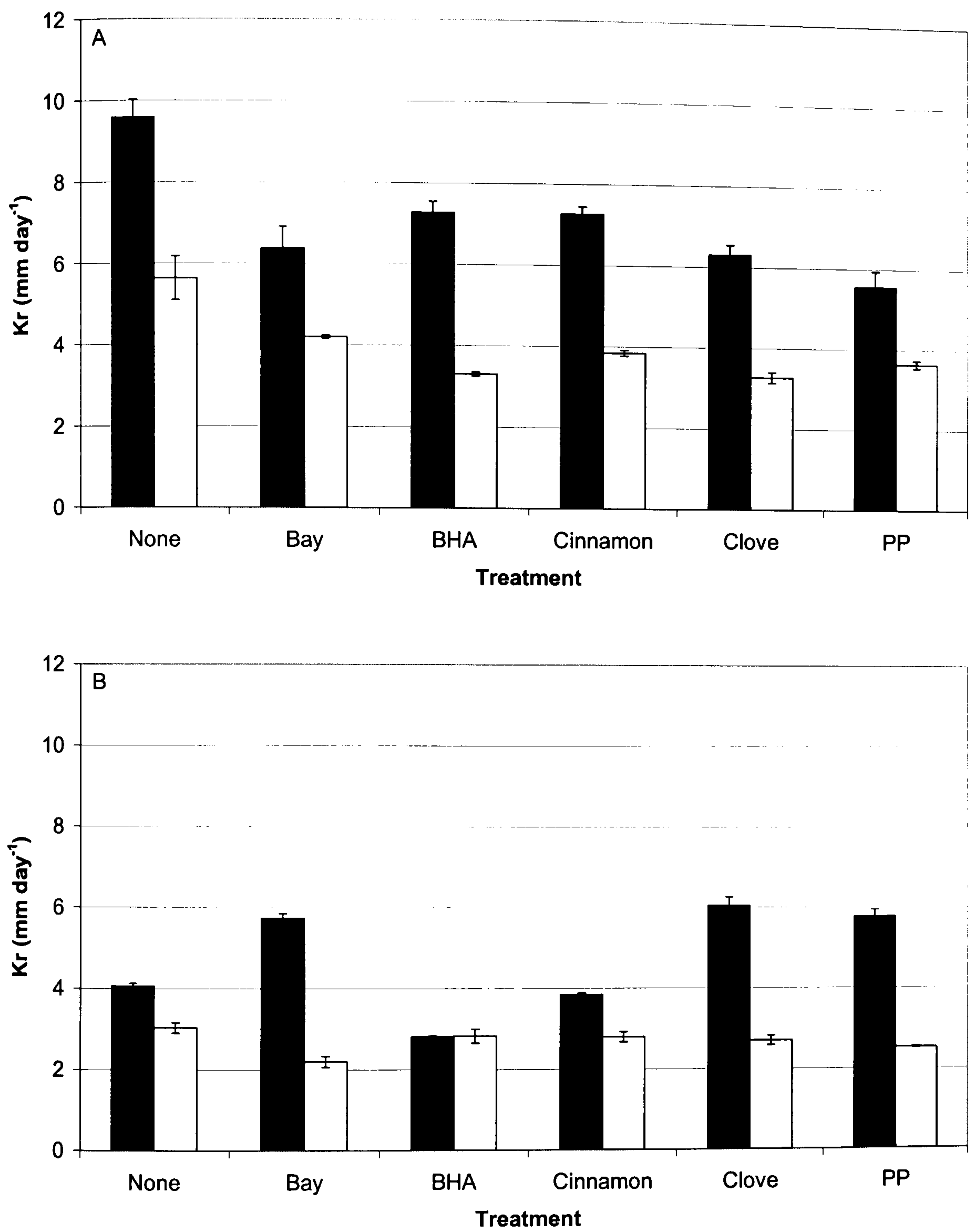


**Figure 3.56** Radial extension rates of *F. culmorum* inoculated on irradiated wheat grain at different temperature (■ 25 °C, and □15 °C),  $a_w$  levels (A. 0.995  $a_w$  and B. 0.955  $a_w$ ) and supplemented with 100 ppm of essential oil or antioxidant. Bars indicate standard error.



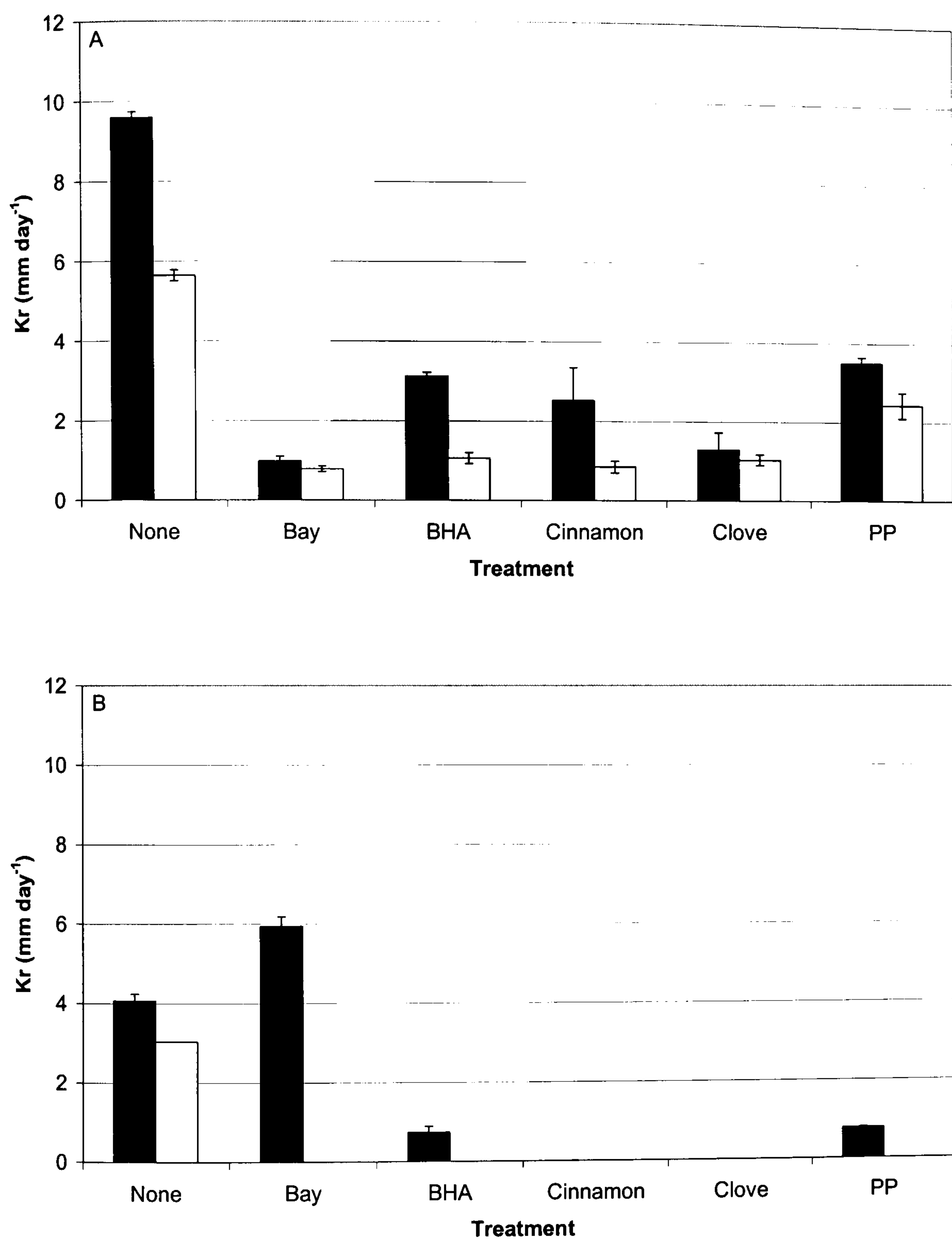


**Figure 3.57** Radial extension rates of *F. culmorum* inoculated on irradiated wheat grain at different temperature (■ 25 °C, and □15 °C),  $a_w$  levels (A. 0.995  $a_w$  and B. 0.955  $a_w$ ) and supplemented with 500 ppm of essential oil or antioxidant. Bars indicate standard error.

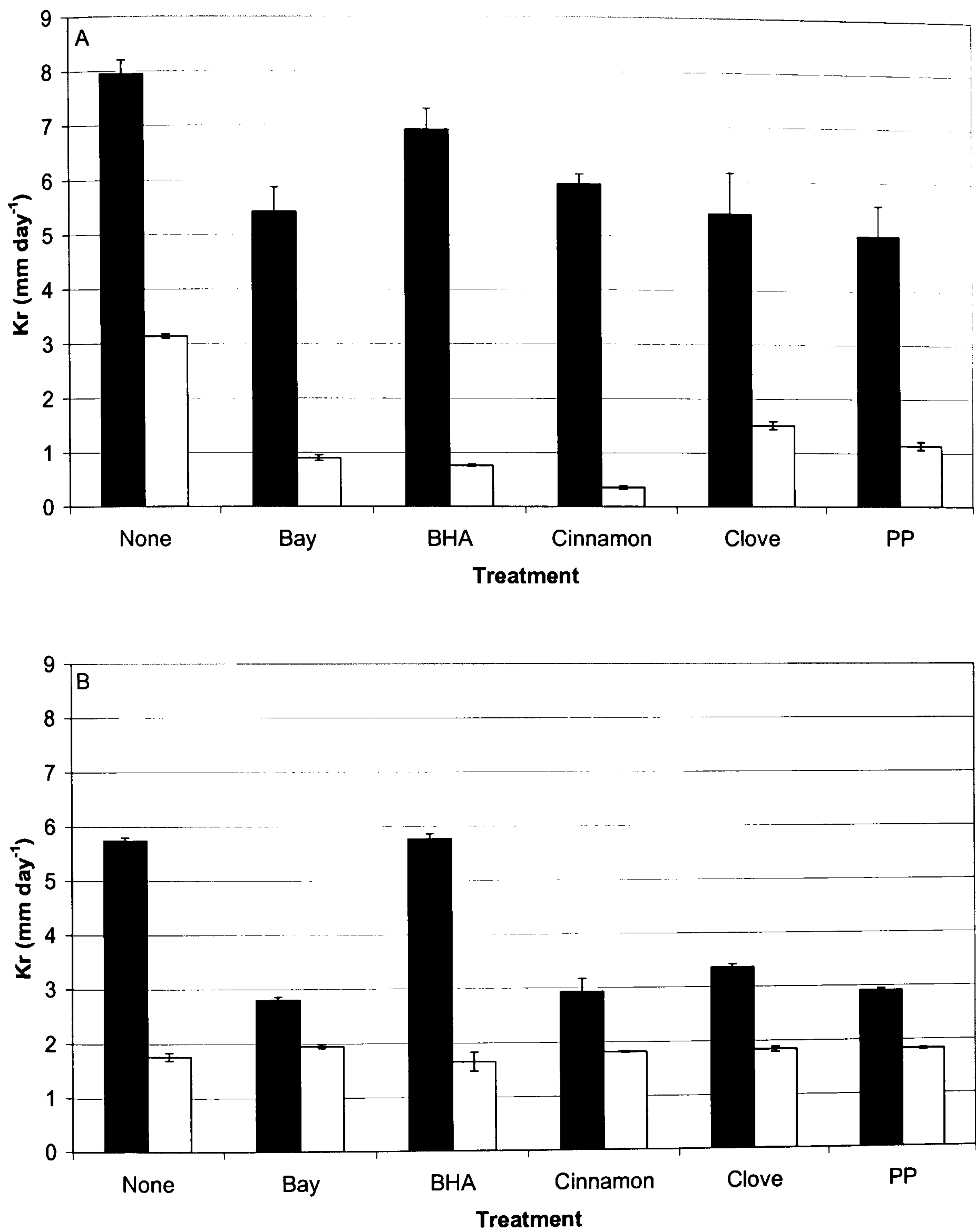


**Figure 3.58** Radial extension rates of *F. graminearum* inoculated on irradiated wheat grain at different temperature (■ 25 °C, and □15 °C),  $a_w$  levels (A. 0.995  $a_w$  and B. 0.955  $a_w$ ) and supplemented with 100 ppm of essential oil or antioxidant. Bars indicate standard error.



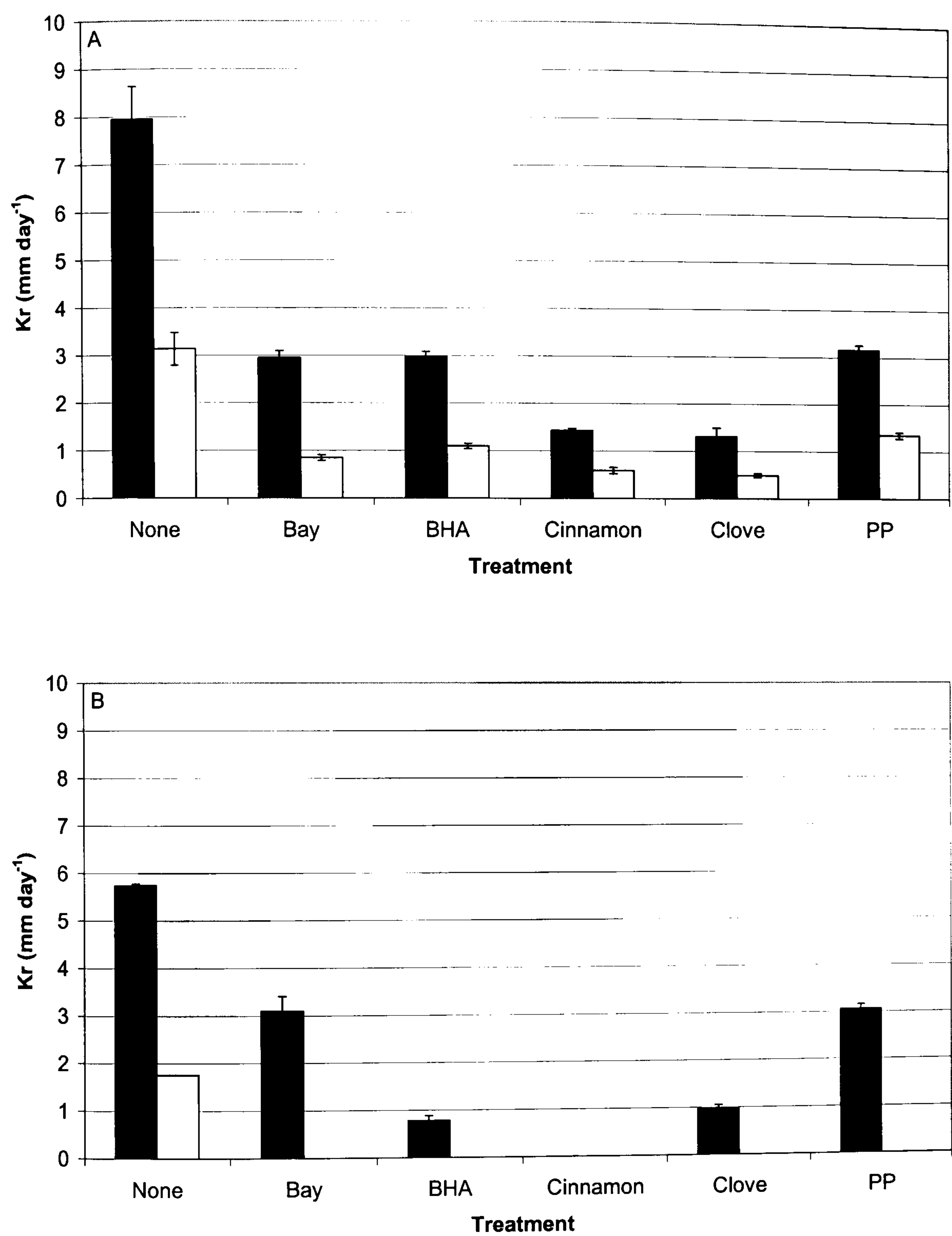


**Figure 3.59** Radial extension rates of *F. graminearum* inoculated on irradiated wheat grain at different temperature (■ 25 °C, and □15 °C), a<sub>w</sub> levels (A. 0.995 a<sub>w</sub> and B. 0.955 a<sub>w</sub>) and supplemented with 500 ppm of essential oil or antioxidant. Bars indicate standard error.



**Figure 3.60** Radial extension rates of *F. poae* inoculated on irradiated wheat grain at different temperature (■ 25 °C, and □15 °C),  $a_w$  levels (A. 0.995  $a_w$  and B. 0.955  $a_w$ ) and supplemented with 100 ppm of essential oil or antioxidant. Bars indicate standard error.





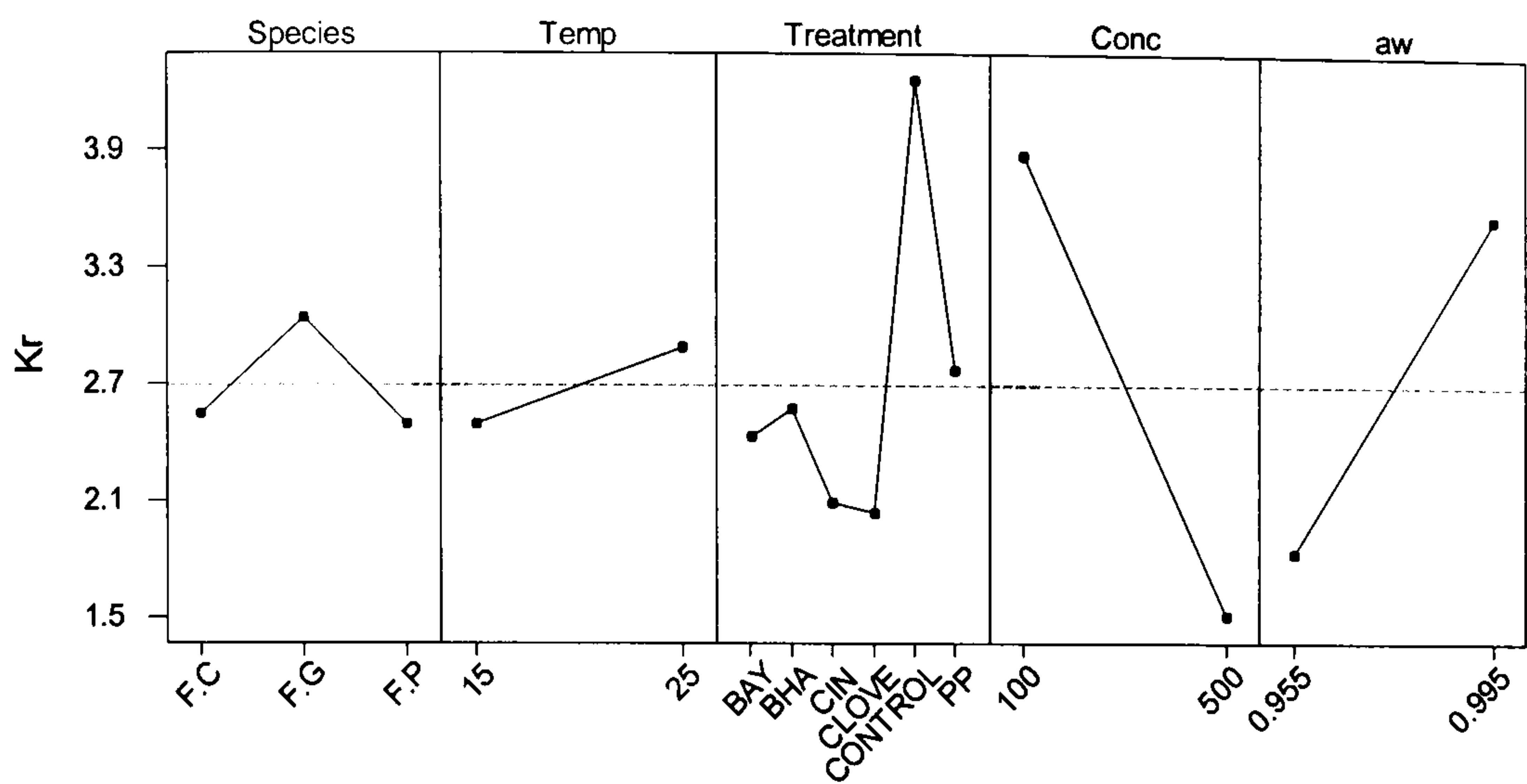
**Figure 3.61** Radial extension rates of *F. poae* inoculated on irradiated wheat grain at different temperature (■ 25 °C, and □15 °C),  $a_w$  levels (A. 0.995  $a_w$  and B. 0.955  $a_w$ ) and supplemented with 500 ppm of essential oil or antioxidant. Bars indicate standard error.

**Table 3.28** *In situ* irradiated wheat grain experiment with essential oil/antioxidant treatments (factors effecting growth). \* Indicates factor elicited a significant effect (P <0.05).

Source of Variation	DF	SS	MS	F	P
a <sub>w</sub>	1	266.9184	266.9184	518.67	<.001*
Conc	1	493.2716	493.2716	958.51	<.001*
Spp	2	176.1668	88.0834	171.16	<.001*
Temp	1	525.2881	525.2881	1020.72	<.001*
Treat	5	323.0902	64.618	125.56	<.001*
a <sub>w</sub> .Conc	1	2.6613	2.6613	5.17	0.023*
a <sub>w</sub> .Spp	2	3.2669	1.6335	3.17	0.043*
Conc.Spp	2	143.6735	71.8367	139.59	<.001*
a <sub>w</sub> .Temp	1	15.3858	15.3858	29.9	<.001*
Conc.Temp	1	22.0844	22.0844	42.91	<.001*
Spp.Temp	2	50.5963	25.2981	49.16	<.001*
a <sub>w</sub> .Treat	5	78.3752	15.675	30.46	<.001*
Conc.Treat	5	49.1747	9.8349	19.11	<.001*
Spp.Treat	10	58.6388	5.8639	11.39	<.001*
Temp.Treat	5	59.5181	11.9036	23.13	<.001*
a <sub>w</sub> .Conc.Spp	2	15.0082	7.5041	14.58	<.001*
a <sub>w</sub> .Conc.Temp	1	13.5935	13.5935	26.41	<.001*
a <sub>w</sub> .Spp.Temp	2	5.976	2.988	5.81	0.003*
Conc.Spp.Temp	2	16.1977	8.0989	15.74	<.001*
a <sub>w</sub> .Conc.Treat	5	39.8758	7.9752	15.5	<.001*
a <sub>w</sub> .Spp.Treat	10	16.2279	1.6228	3.15	<.001*
Conc.Spp.Treat	10	72.7507	7.2751	14.14	<.001*
a <sub>w</sub> .Temp.Treat	5	30.0706	6.0141	11.69	<.001*
Conc.Temp.Treat	5	26.463	5.2926	10.28	<.001*
Spp.Temp.Treat	10	21.0555	2.1055	4.09	<.001*
a <sub>w</sub> .Conc.Spp.Temp	2	4.2528	2.1264	4.13	0.017*
a <sub>w</sub> .Conc.Spp.Treat	10	21.3542	2.1354	4.15	<.001*
a <sub>w</sub> .Conc.Temp.Treat	5	12.8464	2.5693	4.99	<.001*
a <sub>w</sub> .Spp.Temp.Treat	10	25.6577	2.5658	4.99	<.001*
Conc.Spp.Temp.Treat	10	35.1602	3.516	6.83	<.001*
a <sub>w</sub> .Conc.Spp.Temp.Treat	10	12.1206	1.2121	2.36	0.01*
Residual	432	222.318	0.5146		
Total	575	2859.039			



Main Effects Plot - Data Means for Kr



Interaction effects plot – Data Means for Kr

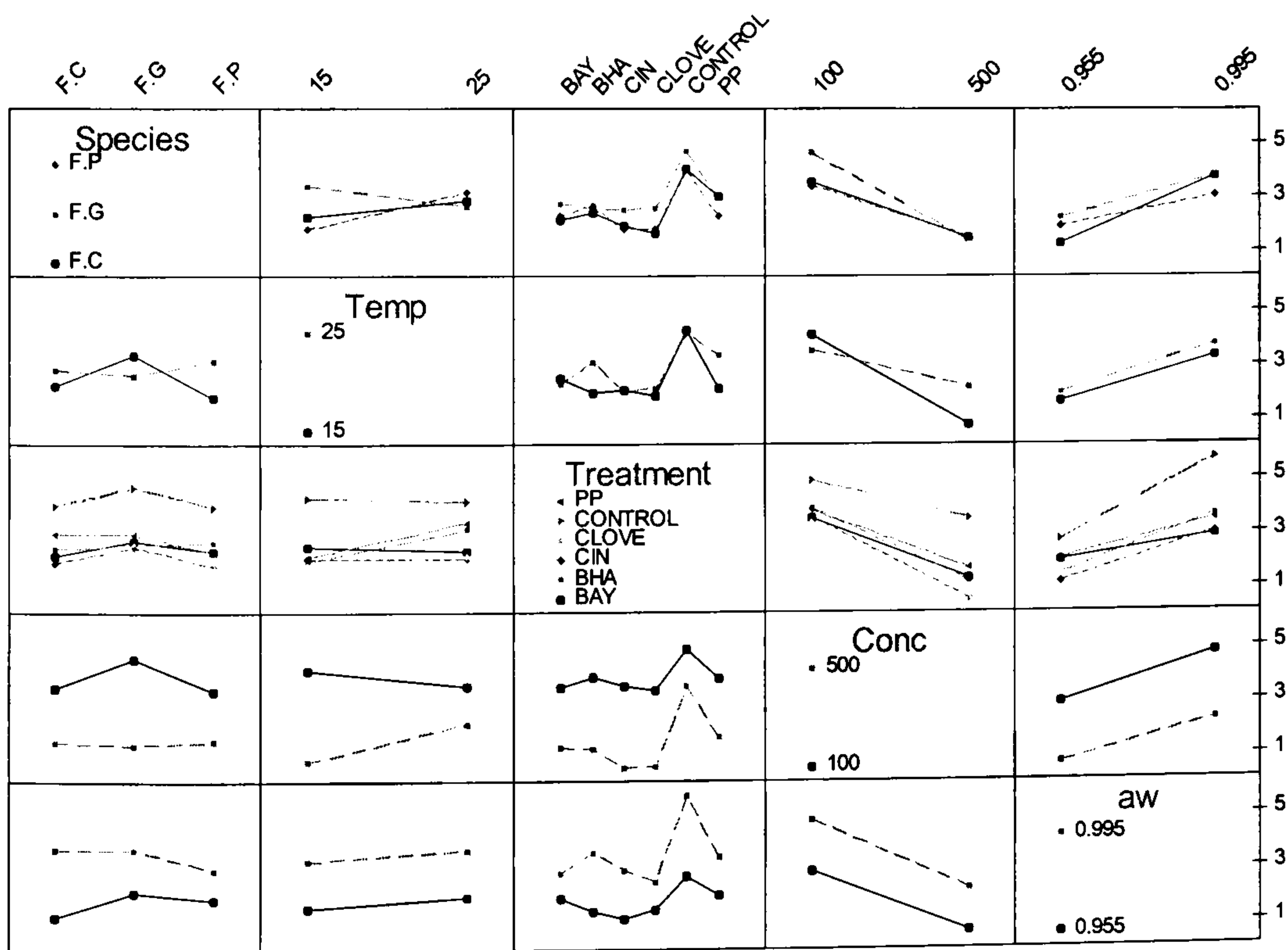


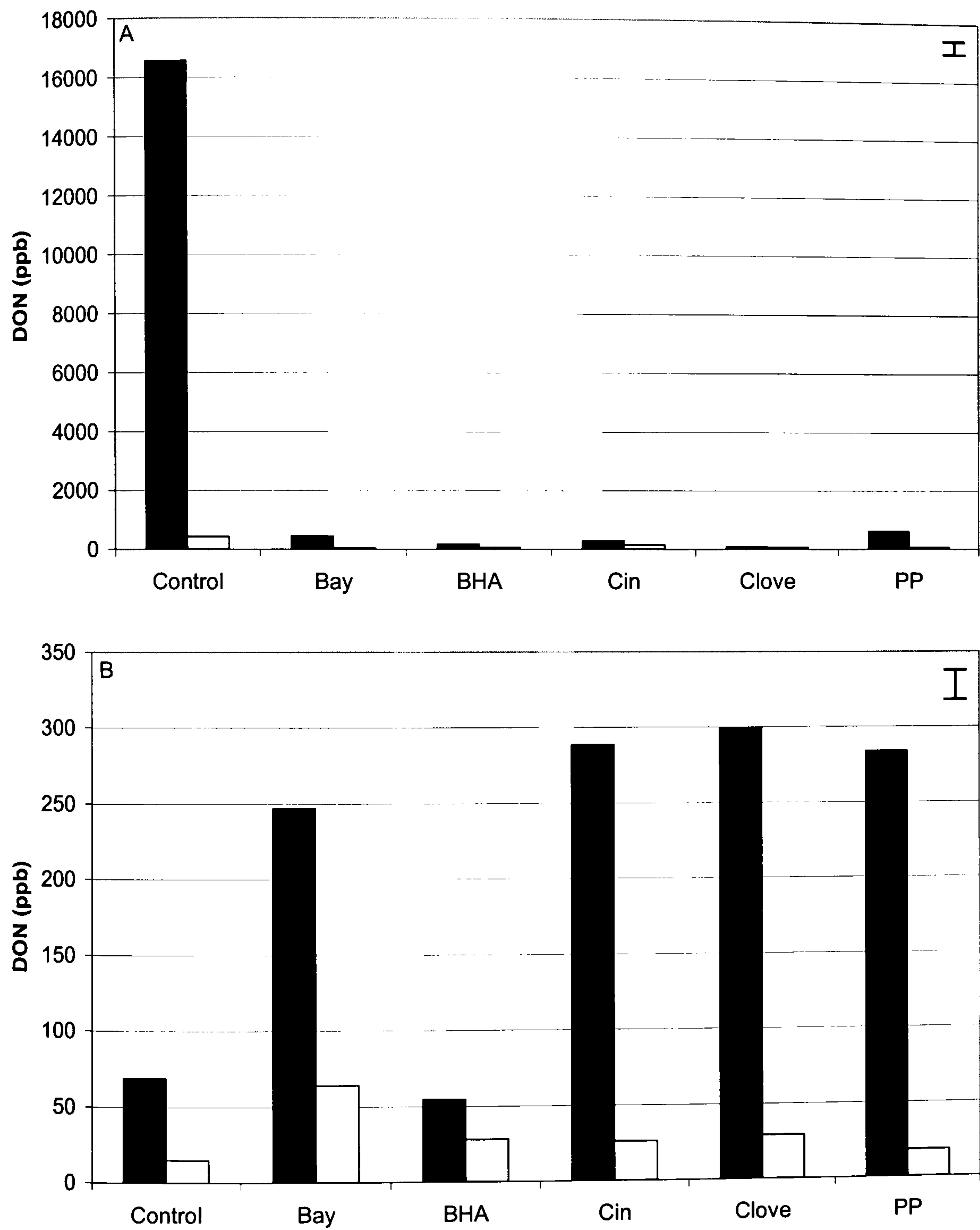
Figure 3.62 Main and interaction effects plots. These plots display the effect on growth rate caused by the different factors, their levels and their interactions. For instance, as  $a_w$  increases Kr also increased.

### 3.8.4 Effect of different essential oils and antioxidants on trichothecene production by *F. culmorum*, *F. graminearum* and *F. poae*

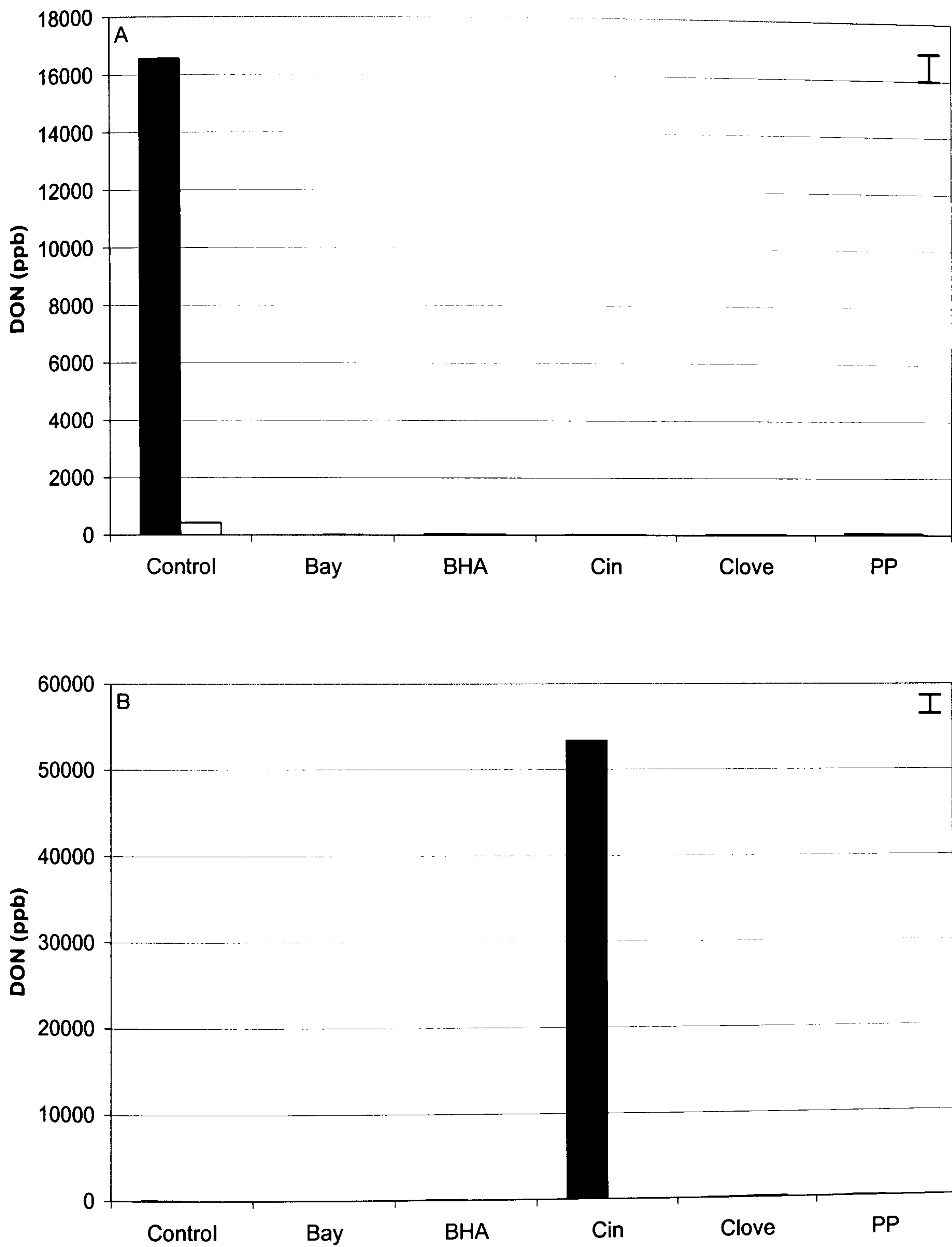
Figures 3.63- 3.70 shows the effect of the treatments on DON and NIV production by *F. culmorum* and *F. graminearum* on irradiated wheat grain. Significant control of DON was achieved by the antioxidants and essential oils, especially at 500 ppm, at both temperatures and  $a_w$  levels tested. 500 ppm of bay leaf oil was most effective at inhibiting DON production under all the temperatures and  $a_w$  levels tested. However, DON production was stimulated above the levels of the control when cinnamon oil or propyl paraben was used at 500 ppm, at 25 °C and 0.955  $a_w$ . At 100 ppm the essential oils and antioxidants were unable to significantly reduce mycotoxin production and in many cases stimulated production. Lower levels of NIV than DON were produced by the *Fusarium* strains in the controls. Control was similar to that for DON under the environmental conditions assayed.

Figures 3.71-3.74 show the effect of the treatments on HT2 and T2 mycotoxin production by *F. poae*. HT2 and T2 production was generally not controlled significantly (> 90 %). Where control was achieved it was normally only under certain temperature,  $a_w$  and treatment conditions. For example, at a concentration of 100 ppm all of the treatments prevented, or significantly reduced mycotoxin production in the 0.955  $a_w$  and 15 °C treatments, but not under any of the other temperature and  $a_w$  conditions. HT2 and T2 production was stimulated under some of the conditions assayed. The most dramatic stimulation was caused by clove oil in the 0.955  $a_w$  and 25 °C samples. ANOVA (See Appendix I Section I.II Table I.11) analysis showed that all of the experimental variables and their interactions had a significant effect on DON, NIV, HT2 and T2 production



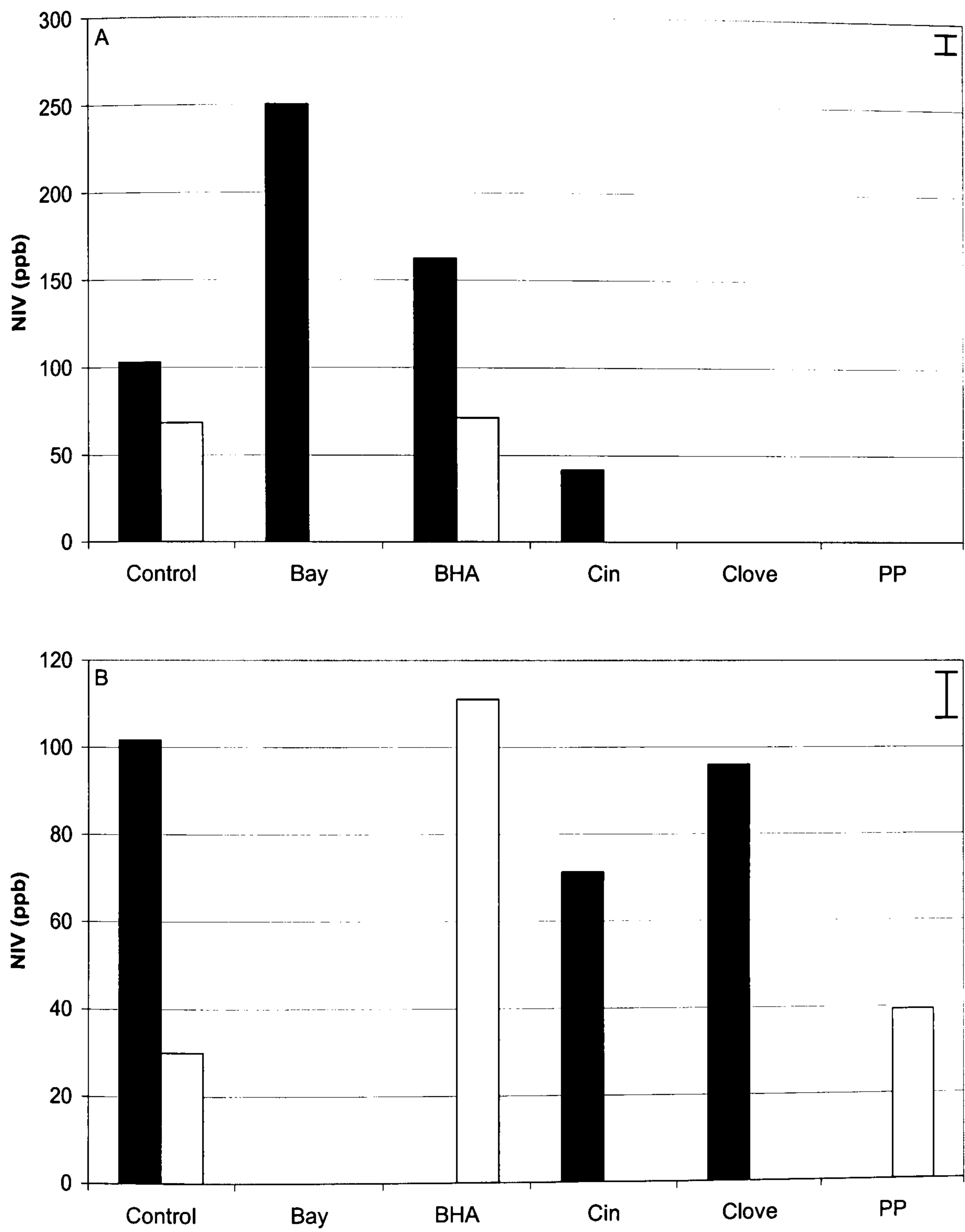


**Figure 3.63** DON production by *F. culmorum* on irradiated wheat grain (A 0.995  $a_w$  and B 0.955  $a_w$ ) treated with 100 ppm of different essential oils or antioxidants. Plates were incubated at ■25 °C or □15 °C. Error bar represents LSD (<0.001 P).

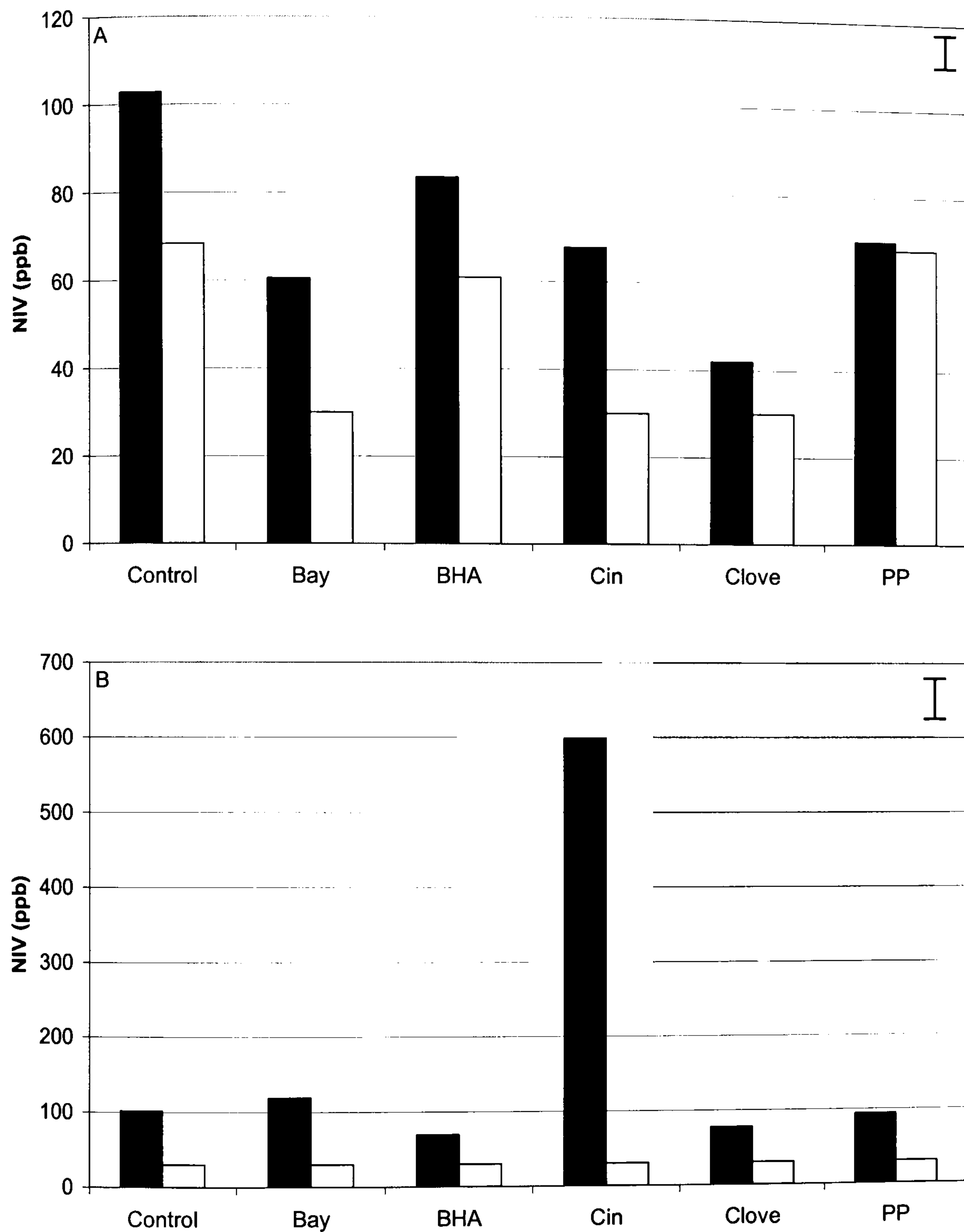


**Figure 3.64** DON production by *F. culmorum* on irradiated wheat grain (A 0.995  $a_w$  and B 0.955  $a_w$ ) treated with 500 ppm of different essential oils or antioxidants. Plates were incubated at ■25 °C or □15 °C. Error bar represents LSD (<0.001 P).



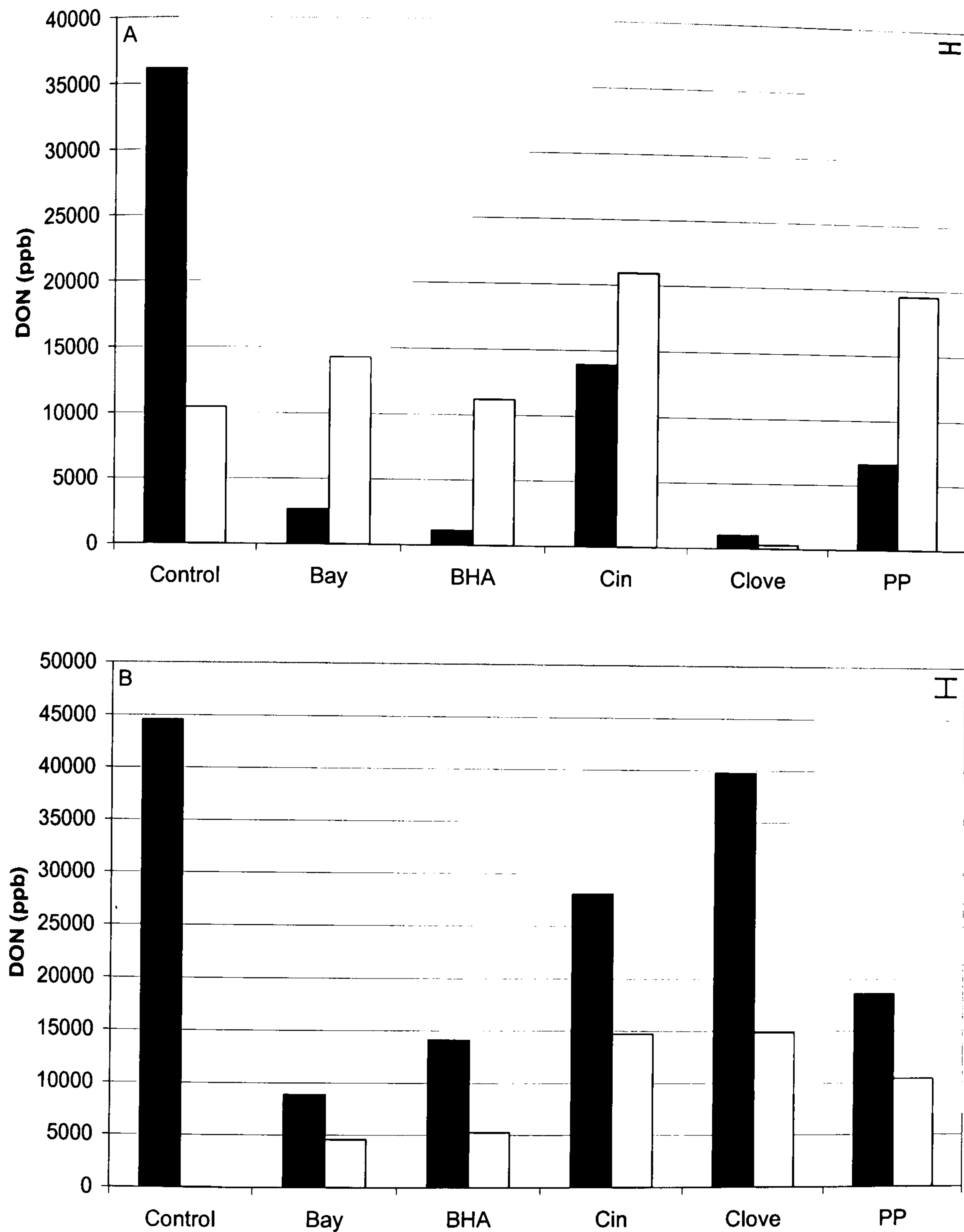


**Figure 3.65** NIV production by *F. culmorum* on irradiated wheat grain (A 0.995  $a_w$  and B 0.955  $a_w$ ) treated with 100 ppm of different essential oils or antioxidants. Plates were incubated at ■25 °C or □15 °C. Error bar represents LSD (<0.001 P).

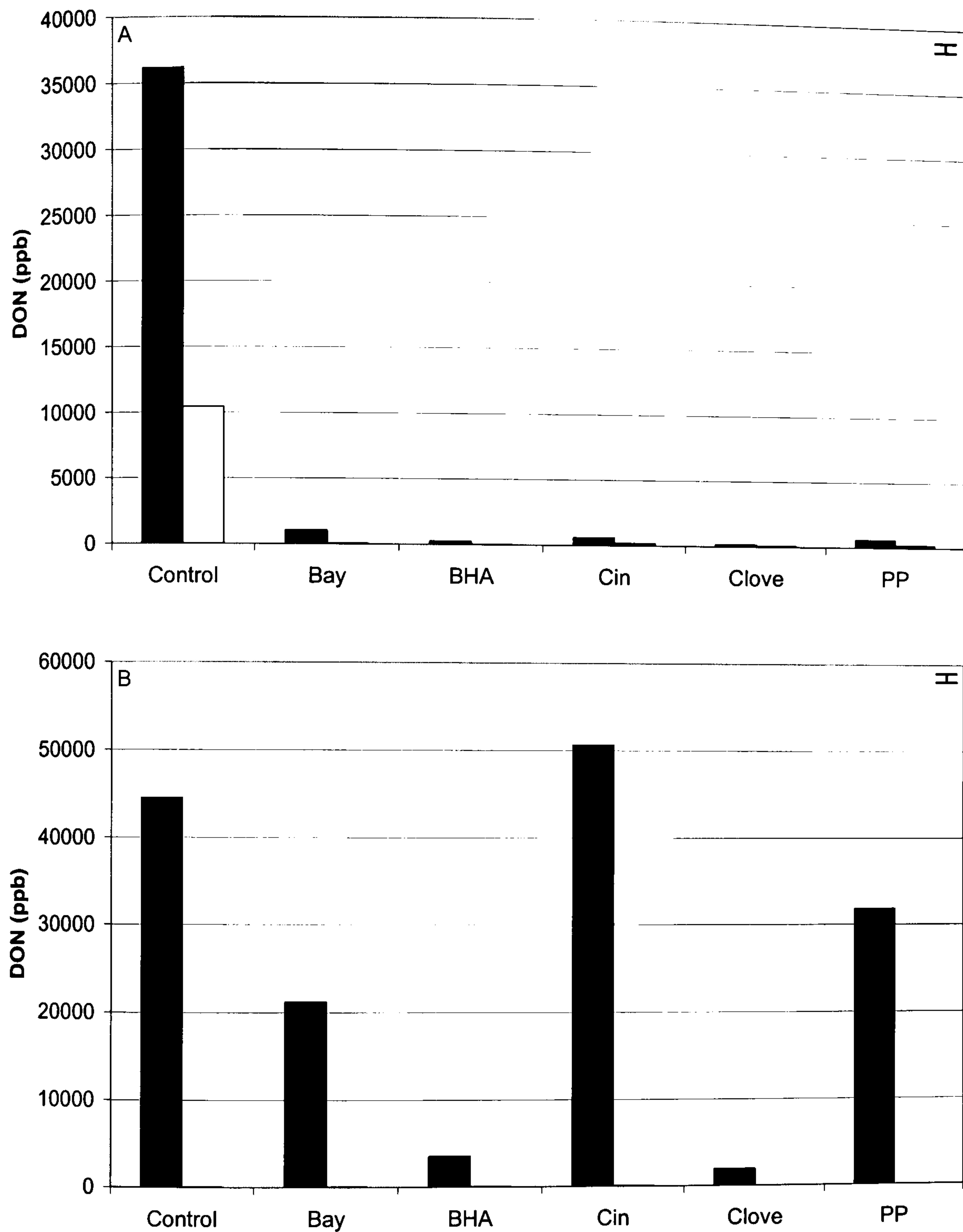


**Figure 3.66** NIV production by *F. culmorum* on irradiated wheat grain (A 0.995 a<sub>w</sub> and B 0.955 a<sub>w</sub>) treated with 500 ppm of different essential oils or antioxidants. Plates were incubated at ■25 °C or □15 °C. Error bar represents LSD (<0.001 P).



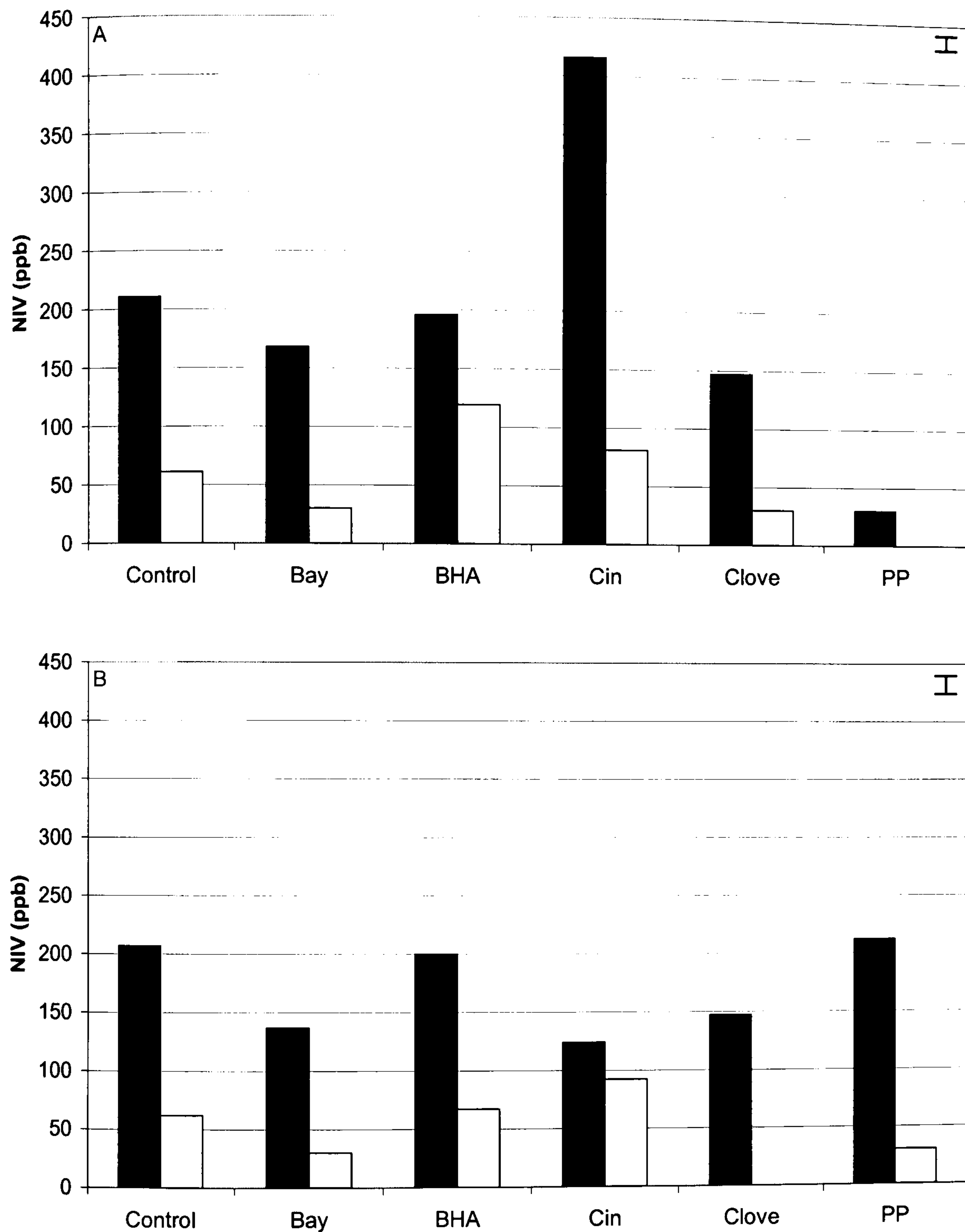


**Figure 3.67** DON production by *F. graminearum* on irradiated wheat grain (A 0.995  $a_w$  and B 0.955  $a_w$ ) treated with 100 ppm of different essential oils or antioxidants. Plates were incubated at ■ 25 °C or □ 15 °C. Error bar represents LSD (<0.001 P).

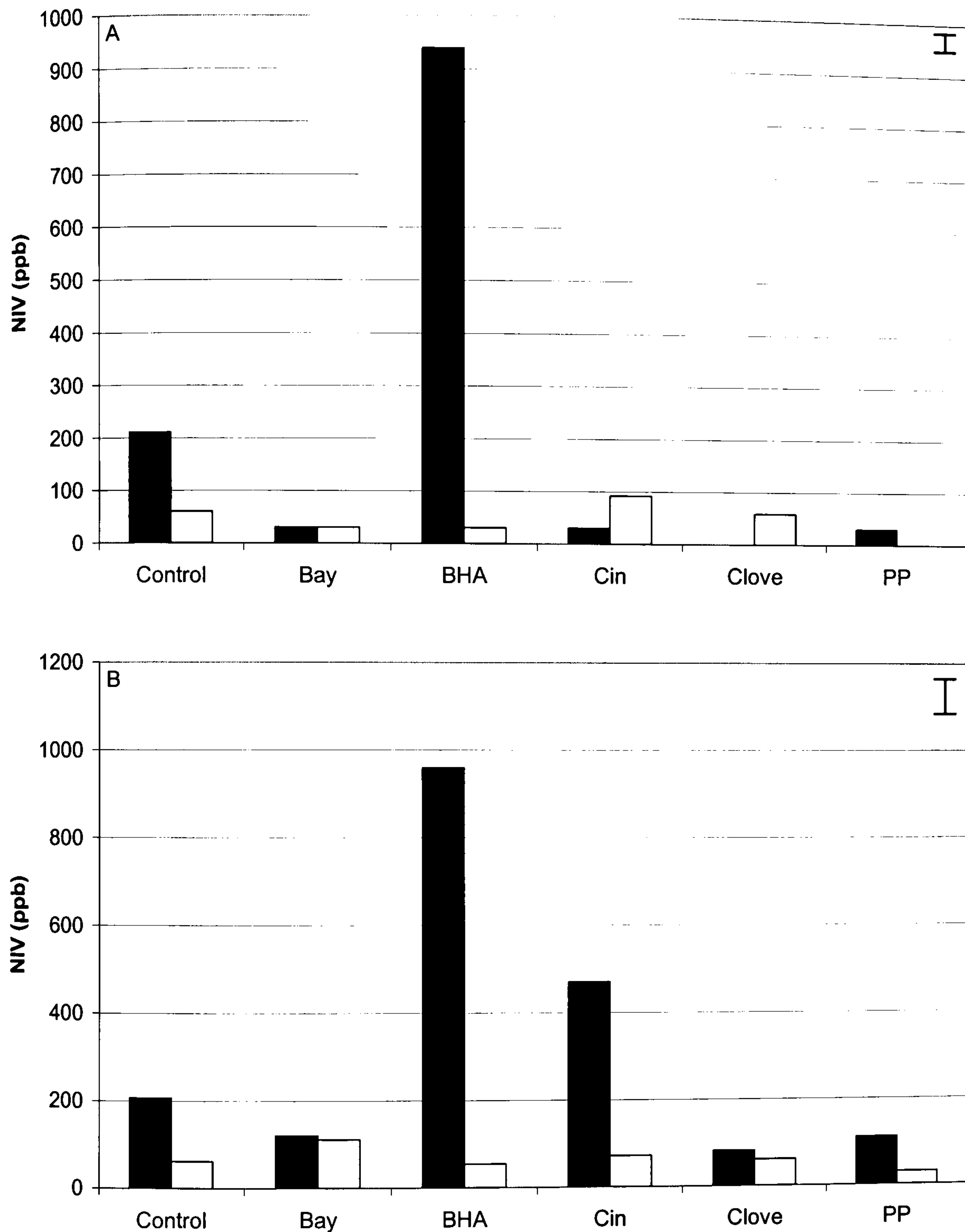


**Figure 3.68** DON production by *F. graminearum* on irradiated wheat grain (A 0.995  $a_w$  and B 0.955  $a_w$ ) treated with 500 ppm of different essential oils or antioxidants. Plates were incubated at ■ 25 °C or □ 15 °C. Error bar represents LSD (<0.001 P).



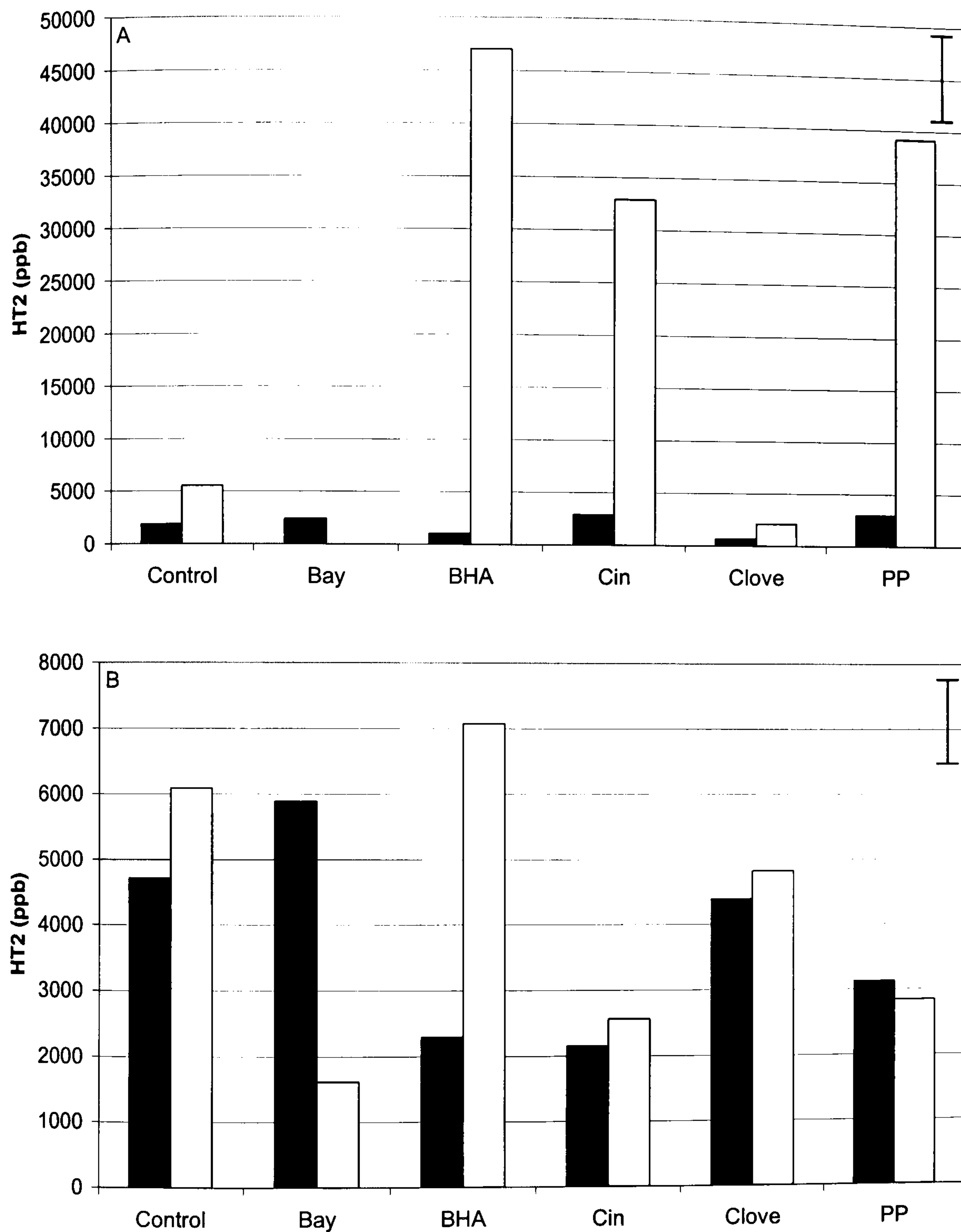


**Figure 3.69** NIV production by *F. graminearum* on irradiated wheat grain (A 0.995  $a_w$  and B 0.955  $a_w$ ) treated with 100 ppm of different essential oils or antioxidants. Plates were incubated at ■ 25 °C or □ 15 °C. Error bar represents LSD (<0.001 P).

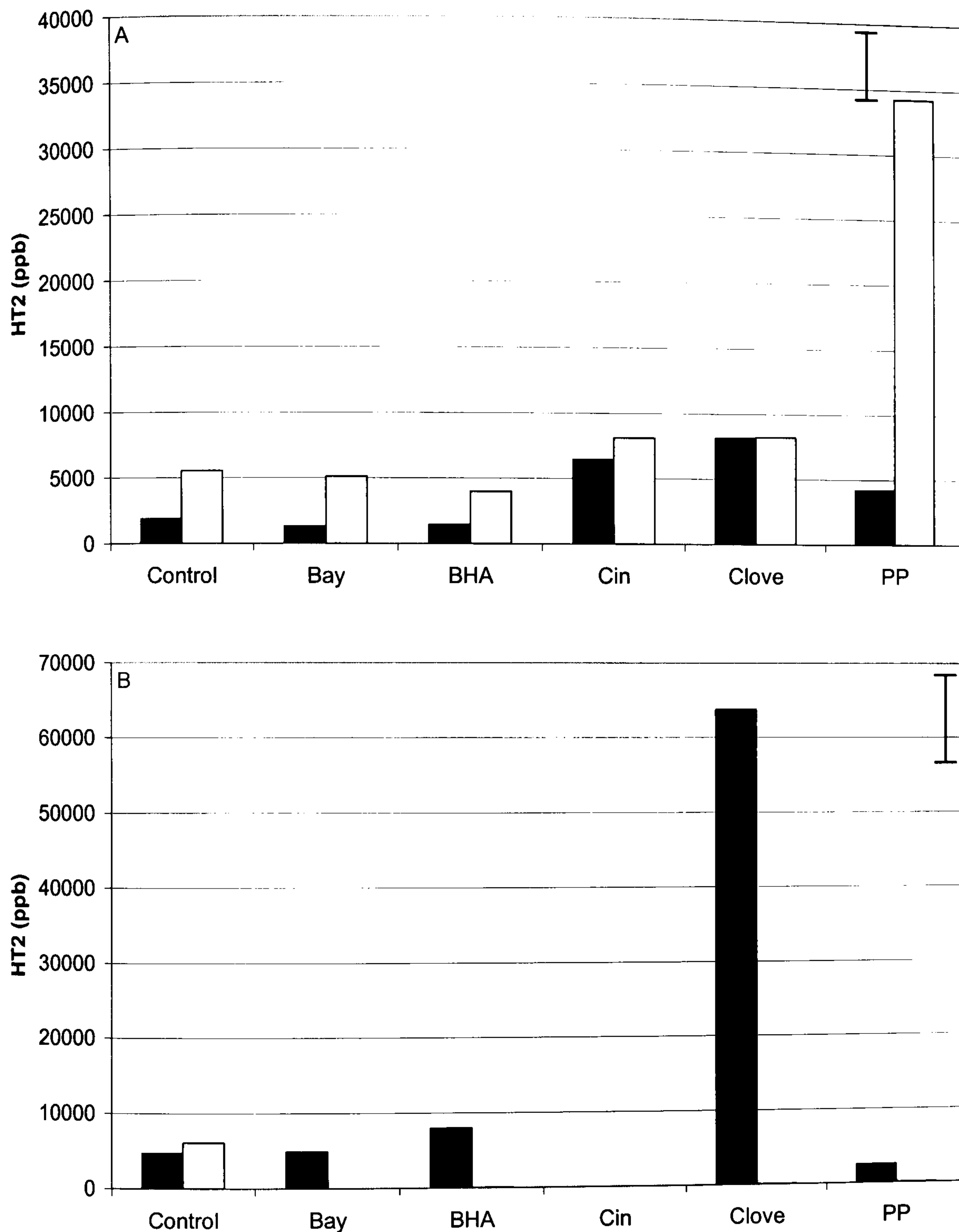


**Figure 3.70** NIV production by *F. graminearum* on irradiated wheat grain (A 0.995  $a_w$  and B 0.955  $a_w$ ) treated with 500 ppm of different essential oils or antioxidants. Plates were incubated at ■ 25 °C or □ 15 °C. Error bar represents LSD (<0.001 P).



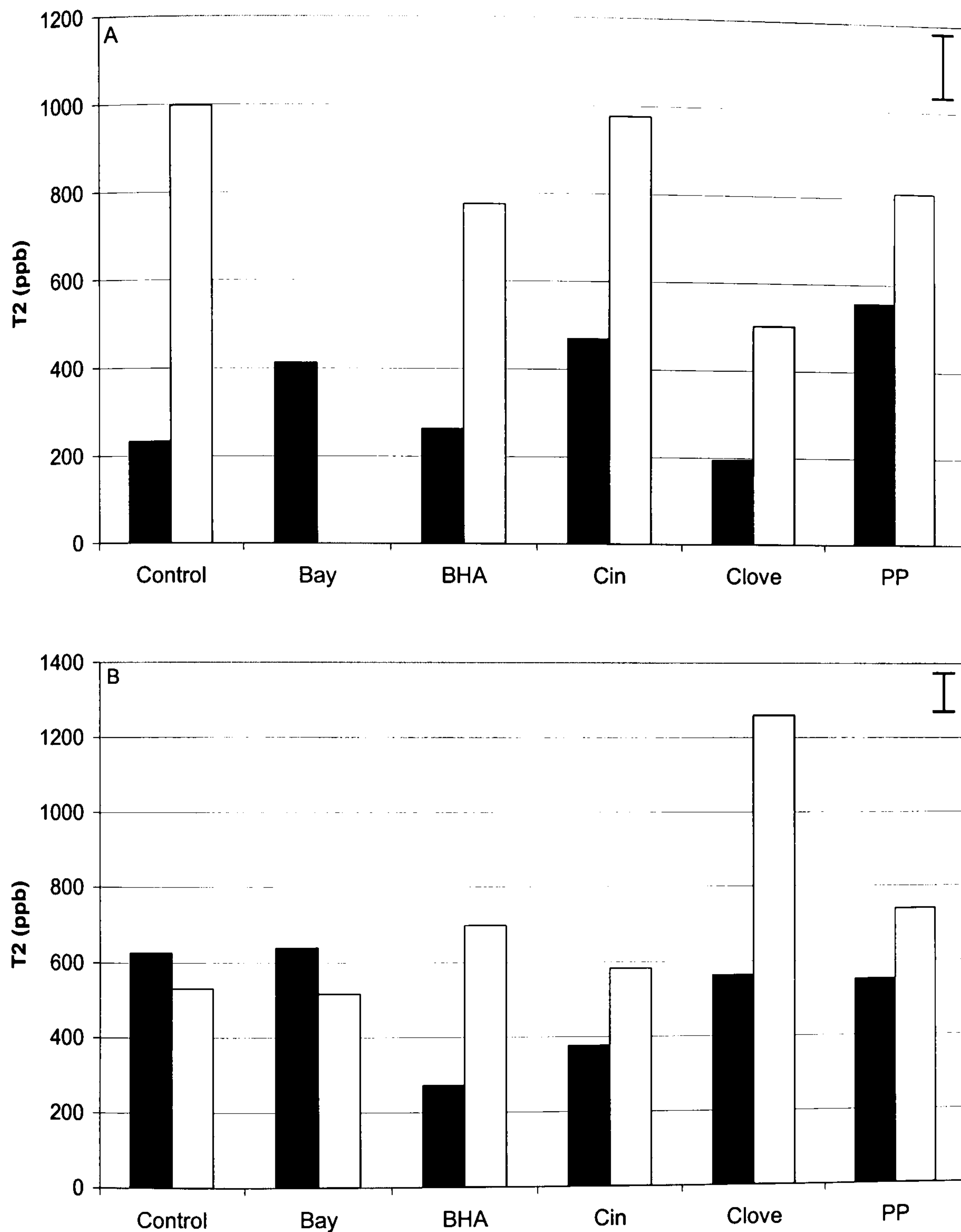


**Figure 3.71** HT2 production by *F. poae* on irradiated wheat grain (A 0.995 a<sub>w</sub> and B 0.955 a<sub>w</sub>) treated with 100 ppm of different essential oils or antioxidants. Plates were incubated at ■25 °C or □15 °C. Error bar represents LSD (<0.001 P).

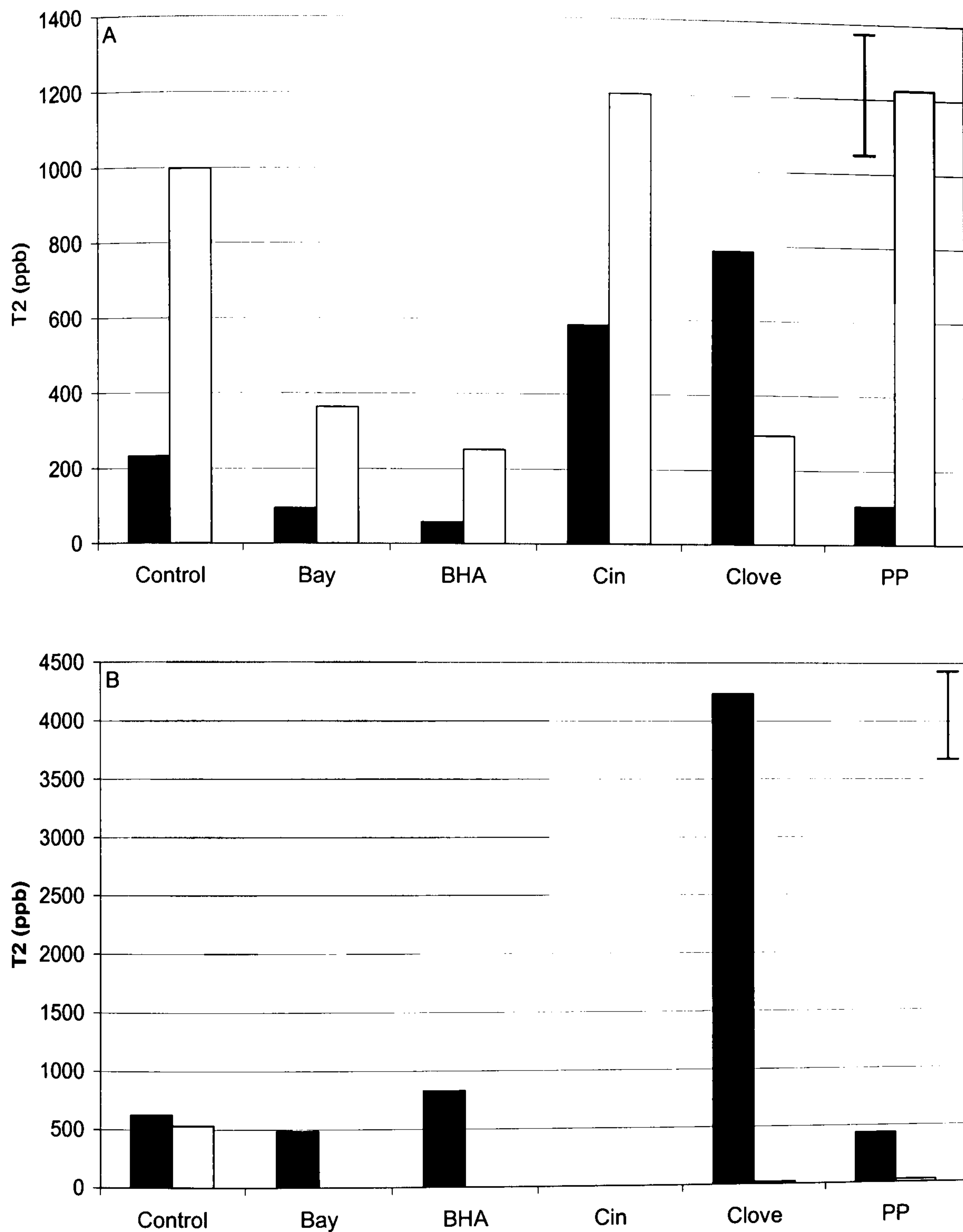


**Figure 3.72** HT2 production by *F. poae* on irradiated wheat grain (A 0.995 a<sub>w</sub> and B 0.955 a<sub>w</sub>) treated with 500 ppm of different essential oils or antioxidants. Plates were incubated at ■ 25 °C or □ 15 °C. Error bar represents LSD (<0.001 P).





**Figure 3.73** T2 production by *F. poae* on irradiated wheat grain (A 0.995 a<sub>w</sub> and B 0.955 a<sub>w</sub>) treated with 100 ppm of different essential oils or antioxidants. Plates were incubated at ■25 °C or □15 °C. Error bar represents LSD (<0.001 P).



**Figure 3.74** T2 production by *F. poae* on irradiated wheat grain (A 0.995 a<sub>w</sub> and B 0.955 a<sub>w</sub>) treated with 500 ppm of different essential oils or antioxidants. Plates were incubated at ■ 25 °C or □ 15 °C. Error bar represents LSD (<0.001 P).



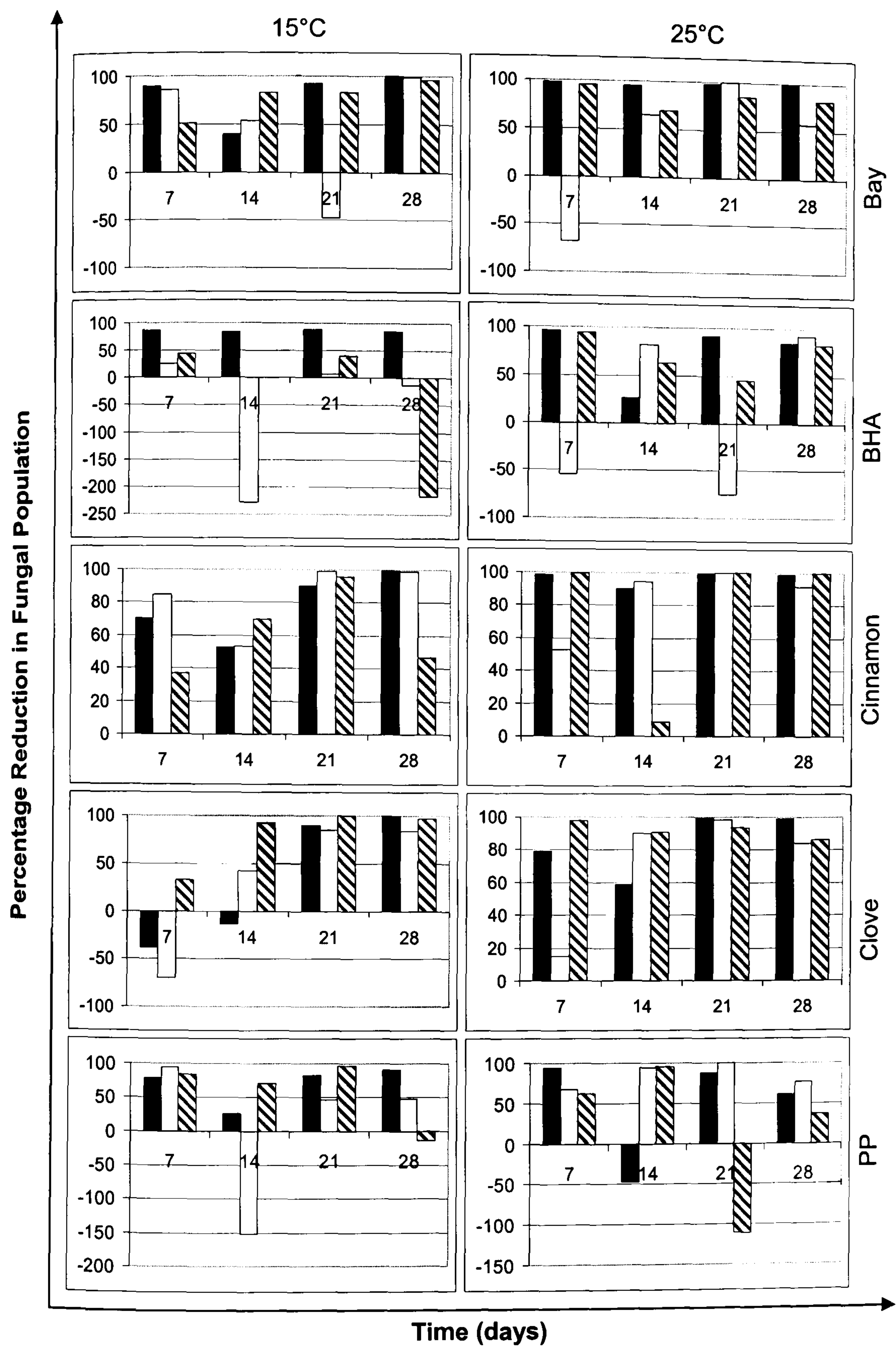
### 3.9 THE EFFECTS OF ESSENTIAL OILS AND ANTIOXIDANTS ON NATURAL FUNGAL POPULATIONS AND ASSOCIATED DON AND NIV LEVELS

In section 3.8 the efficacy of essential oils and antioxidants as controls for growth and toxin production of three *Fusarium* species was tested. In this experiment, the efficacy of the essential oils and antioxidants identified in the previous study were tested in naturally contaminated wheat grain.

The impact of the essential oils or antioxidants on the populations of fungi in naturally contaminated wheat grain was determined by comparing CFU results from wheat grain treated with essential oil or antioxidant with those from the untreated controls. The percentage reduction in fungal populations caused by the different treatments, environmental conditions and time are shown in Figure 3.75. The essential oils and antioxidants were able to inhibit fungal populations by >90 % under the majority of experimental conditions. The PP and BHA were the least effective population control treatments and under some conditions caused stimulation in CFU numbers. The largest stimulation (>200 %) in CFU occurred at 0.95  $a_w$ , 15 °C, after 14 days of incubation with a BHA treatment. Plate 3.2 shows the dramatic difference even one of the less effective treatments has on fungal contamination. In the sample treated with 200 ppm of BHA contamination of the grain is visibly less than in the untreated sample.

Mycotoxin analysis of the natural wheat grain treatments after 28 days incubation showed that DON and NIV were present in the majority of the samples. However, DON was the only toxin present in concentrations greater than 200 ppb.

Figure 3.76 shows the DON results for the samples incubated at 25 °C and 15 °C. Bay essential oil was able to completely inhibit DON production in all of the  $a_w$  and temperature conditions. Stimulation of DON production only occurred with cinnamon essential oil treatment at 0.97  $a_w$  25 °C.



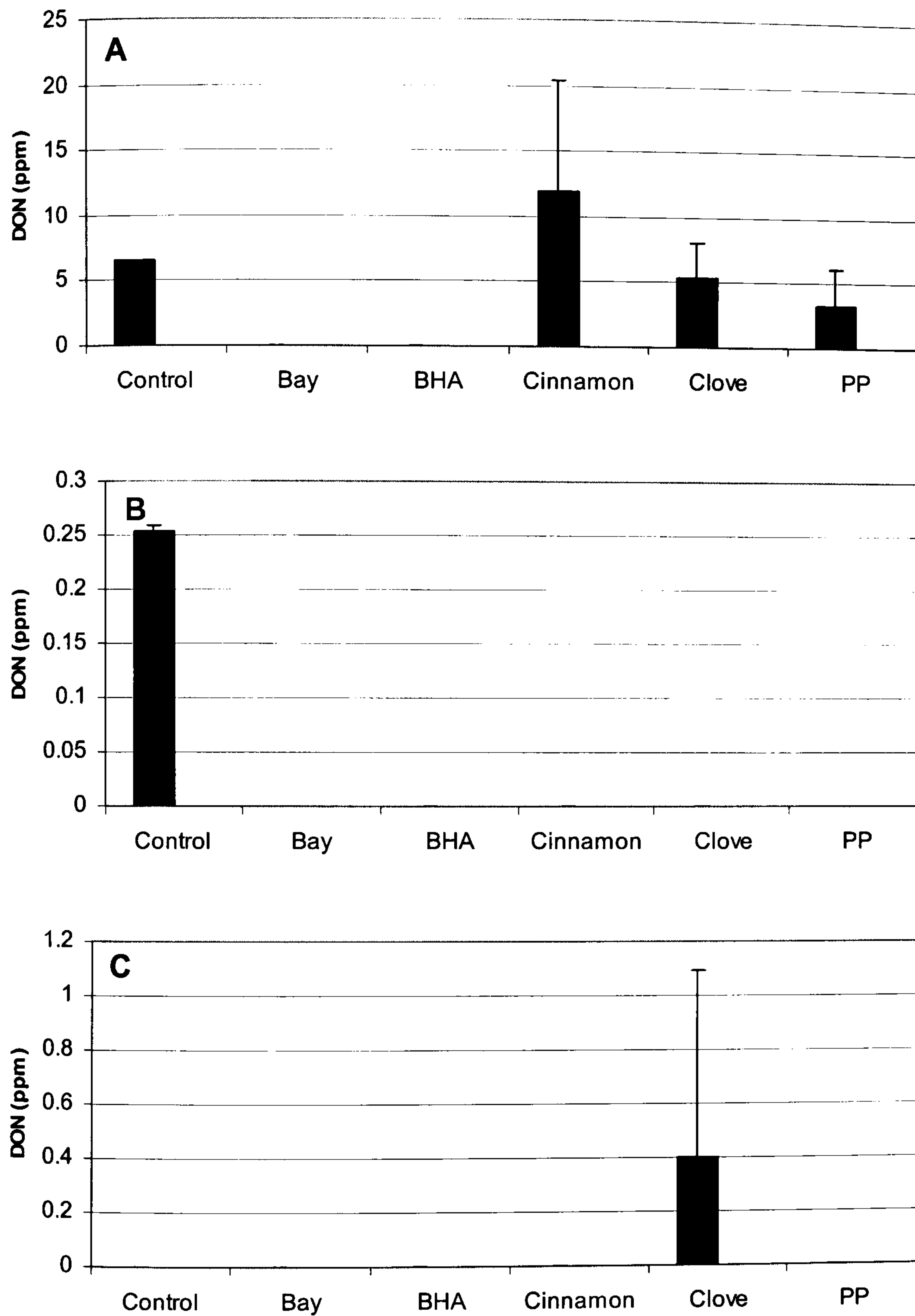
**Figure 3.75** Percentage reduction over time in natural fungal populations of nonsterile wheat grain by essential oil or antioxidant treatment at a concentration of  $200 \mu\text{g g}^{-1}$ . The wheat grain was adjusted to different  $a_w$  levels (■  $0.97 a_w$  □  $0.95 a_w$  and ▨  $0.93 a_w$ ) and incubated at  $25^\circ\text{C}$  and  $15^\circ\text{C}$ .





**Plate 3.2** Fungal contamination in non-sterile wheat grain adjusted to 0.93  $a_w$  and incubated at 25°C for 28 days, untreated (A) and treated with 200ppm BHA (B).





**Figure 3.76** DON present in non sterile grain samples adjusted to different  $a_w$  levels (A. 0.97  $a_w$ , B. 0.95  $a_w$  and C. 0.93  $a_w$ ) after 28 days of incubation at ■ 25 °C and □ 15 °C. Error bars indicate STE.



# CHAPTER 4

## DISCUSSION

#### 4.1 EFFECT OF ENVIRONMENTAL CONDITIONS ON GROWTH OF *F. CULMORUM* AND *F. GRAMINEARUM*

The study of growth rates is important as it provides a good indication of the speed at which a fungus is able to colonise and transverse a substrate. Growth rates may also indicate which species may be dominant over a particular substrate; fast growing species have an advantage over slower species as they can reach and utilise resources before their competitors (Magan & Lacey, 1984a; Marin *et al.*, 1998a; Marin *et al.*, 1998c). Throughout the different experiments colonisation rates of *F. culmorum* were often significantly affected by the various abiotic factors under which it was assayed. The interactions between these factors were often also statistically significant.

##### i). Temperature

Temperature has been extensively documented as being a factor that can significantly affect growth rates of spoilage fungi (Magan & Lacey, 1984b; Marin *et al.*, 1995b; Sautour *et al.*, 2001). Growth of *F. culmorum* isolates used in this work was generally significantly reduced at 15 °C when compared to 25 °C. However, the effects of temperature on relative growth rates were most marked at higher  $a_w$  levels on a wheat agar substrate. When wheat grain substrate or a lower  $a_w$  levels were used, temperature elicited little effect on growth rate. This is similar to the results obtained in a previous study by Magan & Lacey (1984b), who investigated the effects of  $a_w$  and temperature on a number of different *Fusaria* isolated from ears of wheat, including *F. culmorum*. The current study only used temperature levels of 15 and 25°C because previous work has shown *Fusarium* species to grow over the temperature range of 5 - 30°C. The reduced impacts of temperature on growth rate at sub-optimal  $a_w$  conditions is probably due to  $a_w$  being the most significant growth limiting factor.

##### ii). Water activity

In this study *F. culmorum* and *F. graminearum* were able to colonise agar and wheat grain in the  $a_w$  range of 0.9 – 0.995  $a_w$ . Water activity had a significant impact on growth of *F. culmorum* and the other *Fusarium* species in this study. However, its



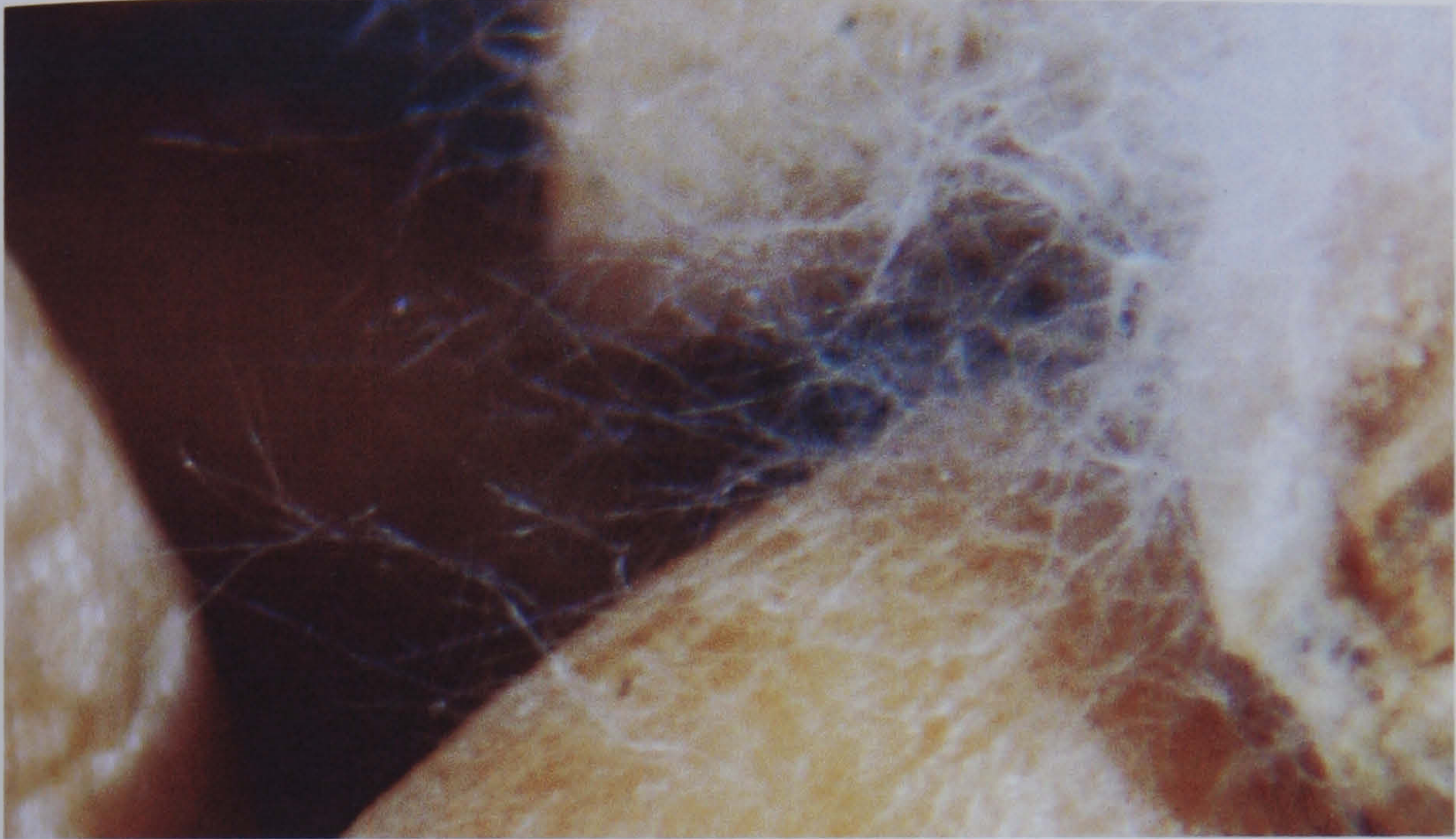
effects are best portrayed in the profile experiment. The highest growth rates for all of the *Fusarium* species tested were attained at 0.995  $a_w$  and 25 °C. Growth rates were generally retarded as the amount of available water was reduced. This trend has also been observed for other *Fusaria* in previous studies (Magan & Lacey, 1984b; Marin *et al.*, 1995a). The growth rates obtained for *F. culmorum* and *F. poae* over the  $a_w$  levels assayed on wheat agar are similar to those observed (Magan & Lacey, 1984b).

### iii). Substrate

The effect of  $a_w$  and temperature was not uniform across substrate types. For instance, on agar the highest growth rates for *F. culmorum* were obtained at 0.995  $a_w$  and 25 °C whereas on wheat grain 0.98  $a_w$  was required for optimal growth rates. The impact of substrate type was similarly noted by Ramos *et al.* (1998), who investigated the growth of *Aspergillus ochraceus* on barley extract agar and barley grain. The effects of  $a_w$  and temperature on *A. ochraceus* were found to be more pronounced when the fungus was grown on the agar medium, than when colonising barley grain.

Comparisons with previously published work are complicated by differences in substrates and fungal strains. This was clearly demonstrated in this work by the *F. culmorum* and *F. graminearum* profiles that show the changes in mycelial colonisation patterns on agar against those on grain. The ability of *F. culmorum* and some of the other species studied to produce floccose aerial mycelium was a distinct advantage when growing on wheat grain. This was because the aerial mycelium allowed the fungi to traverse intergranular spaces (Plate 4.1) with more ease than those species that were unable to produce aerial mycelium (Rayner *et al.*, 1995). However, this advantage provided by aerial mycelium would be reduced in systems where grain is tightly packed together, thus reducing the intergranular air spaces between grains. Also, in a system where the grain is mixed, aerial mycelium may become fragmented, facilitating dispersion of the fungus and thus aiding colonisation. In a mixed system, spore load may probably initially be of greater importance than mycelial growth (Marin *et al.*, 1997a).





**Plate 4.1** Example of aerial mycelium produced by *F. culmorum* bridging intergranular air spaces.



## 4.2 THE EFFECT OF ENVIRONMENTAL CONDITIONS ON DEOXYNIVALENOL AND NIVALENOL PRODUCTION

It has been shown that moisture conditions at anthesis are crucial in determining infection and mycotoxin production by *F. culmorum* and *F. graminearum* on wheat during grain ripening (Magan & Lacey, 1984b; Magan & Lacey, 1988; Lacey *et al.*, 1999). Very few studies have been carried out to determine the effect of key environmental factors such as available water and temperature on mycotoxin production. Some studies have identified the  $a_w$  range for germination and growth of different *Fusarium* species (Magan & Lacey, 1984a; Magan & Lacey, 1984b). However, in these studies the effect of environmental factors on mycotoxin production was not studied. More recently, the effect of  $a_w$  and temperature have been linked to fumonisin production by *Fusarium verticillioides* and *Fusarium proliferatum* infecting maize (Marin *et al.*, 1999a).

The temporal production profiles for DON and NIV (see section 3.2) show the dramatic effects that changes in  $a_w$ , temperature, substrate and time have on toxin production. This study has provided parallel information on growth and DON/NIV production for the first time. Overall, the highest DON levels produced by *F. culmorum* and *F. graminearum* were at 25 °C and 0.995  $a_w$  on wheat agar after 40 days incubation. These conditions were also optimal for growth. In this study whole colonies were harvested, which included a gradation of culture age in a single sample. This may explain the early detection of mycotoxins in some of the treatments. The different  $a_w$  and temperature optimum for NIV compared with DON production was interesting and suggests that *F. culmorum* and *F. graminearum* may respond differently to  $a_w$  and temperature stress, in terms of toxin production. The fungus may produce NIV under sub-optimal growth conditions for improving competitiveness. While both fungi produce less NIV than DON, it should be borne in mind that the former is more toxic than the later.

Previous small-plot field-based studies with *F. culmorum* using misting have demonstrated that in the UK infection and mycotoxin production was highest during early anthesis when conducive humidity periods exist for infection (Lacey *et al.*, 1999). Surprisingly, Brizele *et al.* (2000) suggested that DON was produced by *F. culmorum* at 0.80  $a_w$  in wheat grain. This is a condition under which *F. culmorum* and most other *Fusaria* are unable to grow (Magan & Lacey, 1984b). This suggests that the accuracy of the  $a_w$  measurements may be doubtful in their study.

In summary, these experiments show that the range of  $a_w$  conditions for DON and NIV production is much narrower than the range for growth. This information is very useful in monitoring critical control points, as it provides the necessary data on the threshold key environmental conditions for growth and mycotoxin production by *F. culmorum* and *F. graminearum*.

### 4.3 INTERSPECIFIC INTERACTIONS AND THEIR IMPACT ON GROWTH AND MYCOTOXIN PRODUCTION

FEB can be caused by a complex of *Fusaria* and other species. In addition to the FEB causing species, filamentous fungi, yeasts and bacterial species that commonly inhabit wheat are likely to be present. Thus, interactions between fungal species inevitably occur, a fact that has been shown extensively in previous studies (Lee & Magan, 1999b; Lee & Magan, 2000; Edwards *et al.*, 2001; Marin *et al.*, 2001). Interspecific interactions have also been shown to be affected by environmental factors, such as  $a_w$ , temperature and the nutritional composition of the substrate. Thus, it is important to understand how *F. culmorum* interacts with other fungal species and the impact of these interspecific interactions on *F. culmorum* and *F. graminearum*.

Spoilage fungi employ different types of primary and secondary strategies when colonising their ecological niche. Strategies used may be combative (C-selected), stress (S-selected) or ruderal (R-selected) or merged secondary strategies (C-R, S-R, C-S, C-S-R) (Cooke & Whipps, 1993). Primary resource capture of grain is influenced by the germination rate, growth rate, enzyme production and the capacity



for sporulation. Subsequent interactions between spoilage fungi result in combat, antagonism and niche overlap which all influence secondary resource capture (Magan *et al.*, 2003).

In this section the impact of interactions on growth and mycotoxin production will be discussed. The effect of  $a_w$ , temperature and substrate on these systems will also be covered. The niche overlap study will be discussed as a method for identifying fungal interactions. This, and analysis of key hydrolytic enzymes, is to some degree indicative of the relative competitiveness *in situ*.

*i). Interaction scores and indices of dominance*

The scoring method used in the current study had been used successfully in a number of previous studies (Magan & Lacey, 1984a; Lee & Magan, 2000; Marin *et al.*, 2001). In the current study *F. culmorum* was able to inhibit or dominate most non-*Fusaria* at the higher  $a_w$  levels and temperature tested on both agar and wheat grain. Under the same conditions against other *Fusarium* species, mutual inhibition or dominance of *F. culmorum* in interactions occurred. At reduced  $a_w$  levels and cooler temperature (0.955  $a_w$ , 15 °C) *F. culmorum* often lost its competitive advantage over the non-*Fusaria*. For instance, at 0.995  $a_w$  and 25 °C on wheat agar *F. culmorum* was able to dominate the ochratoxigenic *P. verrucosum* on contact, but when the conditions were changed to 0.955  $a_w$  and 15 °C mutual inhibition on contact resulted.

The effect of temperature on interactions was most apparent on the wheat agar. Comparison of the total  $I_D$  scores show that *F. culmorum* was dominant at 25 °C, whereas at 15 °C it was not. Interactions between *F. culmorum*, *M. nivale* and *M. nivale* var. *majus* were most affected by temperature. At 25 °C, *F. culmorum* dominated both of the *Microdochium* species, whereas at 15 °C the *Microdochium* species were able to dominate *F. culmorum*. The reason why the *Microdochium* species are able to compete better than *F. culmorum* at 15 °C is that this temperature is closer to the former species optimum for growth. The ability of *Microdochium* species to inhibit *F. culmorum* could explain why a recent study by Pirgozliev (2002) found that reduction of *Microdochium* species in a FEB complex resulted in an increase in the *Fusarium* populations.

Substrate had a significant effect on interactions. *F. culmorum* was generally less dominant on agar than on grain. This may have been due to extracellular metabolites (important in primary resource capture) that were able to diffuse more easily in an agar substrate than in grain, therefore being more important within the agar system; and *F. culmorum*'s ability to bridge intergranular air spaces with aerial mycelium, enabling the fungus to colonise a grain more effectively.

Interestingly, *F. culmorum* was never able to achieve dominance over *F. graminearum* regardless of the conditions, and they were mutually antagonistic to each other at 0.955  $a_w$  on agar. This supports published work reporting FEB to be increasingly resulting from *F. graminearum* inoculum and infection in the UK (Edwards *et al.*, 2001; Bateman & Murray, 2001).

The dark pigmentation of colony perimeters in the proximity of a competing species during interaction implicates phenol-oxidizing activity as a contributor to the interaction responses. Also the promotion and suppression of aerial mycelium production has been linked to the enzymes tyrosinase and laccase respectively (Rayner *et al.*, 1995).

#### ii). Impact of interactions on growth of *F. culmorum*

Interactions had a variable effect on the colonising ability of *F. culmorum*. In some instances growth was significantly increased (i.e. *F. culmorum* vs. *A. tenuissima* at 25 °C and 0.995 and 0.955  $a_w$ ), and in others growth was significantly reduced (i.e. *F. culmorum* vs. *F. graminearum* 25 °C, 0.995  $a_w$ ). However, changes in growth rate did not seem to correlate with changes in interaction type. For instance, *F. culmorum* was dominant on contact with *A. tenuissima* in all of the conditions assayed yet at 25 °C growth of *F. culmorum* was significantly stimulated by this interaction whereas at 15 °C growth was not significantly different from that of the homogenous control.

Earlier interaction studies by Marin *et al.* (1997a) investigated the effects of *Fusarium moniliforme* and *Fusarium proliferatum* on four maize grain spoilage fungi. They compared the relative growth rates of the different fungi, populations (CFUs) and percentage grain infection with the interaction scores. It was concluded that there was no correlation between these results even though there were significant differences



between the treatments. However, it should be noted that unlike the current study only the interaction scores were determined in dual cultures, thus making comparisons with this work more difficult. Magan & Lacey (1984a) also compared interaction scores with growth rates determined homogenously on agar substrates. Again it was found that there was no clear correlation between dominance and growth rate. Both studies concluded that growth was a factor in competitiveness but could not be the only factor used to determine dominance.

In a more recent study on maize grain concerning spoilage fungal interactions, growth rates of the fungi were determined in dual culture (Lee & Magan, 2000). The growth rate of *Aspergillus ochraceus* was mainly reduced by the presence of competing species. No stimulation of growth was observed for *A. ochraceus* under any of the assayed conditions as was seen for *F. culmorum* in the current study.

### iii). *Impact of interactions on mycotoxin production*

This is the first study on the impact of interactions in combination with environmental stress ( $a_w$  and temperature) on DON and NIV production by *F. culmorum*. Incubation in dual culture had a significant effect on DON production. In the majority of cases DON was significantly reduced or completely inhibited. NIV levels were generally unaffected by dual culture interactions. However, DON and NIV levels were occasionally stimulated above that of the homogenous control. The interaction types themselves did not have a significant effect on mycotoxin production, i.e. dominance of *F. culmorum* did not necessarily result in a corresponding mycotoxin response. For instance, on wheat agar *F. culmorum* vs. *M. nivale* resulted in mutual inhibition on contact and dominance on contact by *F. culmorum* at 0.995  $a_w$  and 0.98  $a_w$  and 25 °C respectively. However, under both of these conditions DON and NIV production was inhibited. Temperature,  $a_w$  and substrate all had a significant impact on mycotoxin production in mixed culture. Variation between replicates was sometimes large, however, particularly on irradiated wheat grain. This may partially have been due to the use of point inoculations rather than inoculating the entire plate with a mixed inoculum of both fungi. Overall, stimulation of DON or NIV production by *F. culmorum* when interacting with other fungal species did not correlate with stimulation of *F. culmorum* growth.



Changes in mycotoxin levels occurring when interactions remain the same may indicate a change in combative strategy by the fungi in response to environmental stress. For example, *F. graminearum* was able to dominate *F. culmorum* on wheat grain at 0.995  $a_w$  and 0.955  $a_w$ . However, at the higher  $a_w$  level DON production was completely inhibited, whereas at 0.955  $a_w$  DON levels were stimulated, indicating that a physiological change had occurred in response to the ecological pressures, even though the interaction outcome remained the same. Other studies have also noted the lack of correlation between interaction type and mycotoxin production, but did not hypothesise as to why this occurred (Marin *et al.*, 1998d; Lee & Magan, 2000). It has been suggested that when some fungi are in close proximity to each other, mycotoxin production is stimulated; this is thought to be because the fungi are trying to pre-emptively exclude other competitors (Lee & Magan, 1999a).

The effect of  $a_w$ , temperature and interspecific interactions on mycotoxin production has previously been observed for other mycotoxins and fungal species (Ramakrishna *et al.*, 1996a; Ramakrishna *et al.*, 1996b; Marin *et al.*, 1998d; Lee & Magan, 1999a; Lee & Magan, 2000; Velluti *et al.*, 2000). However, in these previous studies on interactions none investigated impacts on DON or NIV production, making direct comparison with the results of this study difficult. However, comparisons with the trends found in those studies can be drawn.

For example, Ramakrishna *et al.*, (1996b) found that T2 toxin production by *F. sporotrichioides* was generally inhibited when grown in dual culture with *Aspergillus flavus*, *P. verrucosum* or *Hyphopichia burtonii* on barley grain. However, as in the current study some stimulation of toxin production was seen under certain conditions.

Marin *et al.* (1997; 2001) showed the effect of competing fungi on fumonisin production by *F. moniliforme* (= *F. verticillioides*) and *F. proliferatum* on maize grain. They found that interaction of *F. moniliforme* (= *F. verticillioides*) with *A. niger*, *A. ochraceus* or *A. flavus* at 15/25 °C and 0.98  $a_w$  resulted in a significant increase in fumonisin production. However, when *F. moniliforme* was grown in dual culture with *Penicillium implicatum* a decrease in fumonisin production occurred. Contrary to the current study, some correlation was found between growth rate and



mycotoxin production. However, this was a comparison with growth rates determined in homogenous culture, and not in dual culture as in the current study.

*iv). Niche overlap indices (NOIs)*

Niche overlap indices constructed from carbon source utilisation profile assays are an alternative method for identifying the competitiveness of fungal species. Niche overlap indices compare the carbon sources that are able to be metabolised by different species. If either of the fungi has 90% or more compatible carbon sources, they will occupy the same nutritional niche (Wilson & Lindlow, 1994; Wilson & Lindlow, 1994). A fungus is assumed to attempt to exclude or inhibit other fungi and microorganisms which inhabit the same nutritional niche. Therefore it is expected that species that produce dominance or inhibition interspecific interactions when grown in dual culture with each other will occupy the same niche, as species utilising the same carbon sources are most likely to have evolved competitive strategies to exclude each other. Fungi that produce the more passive interactions (e.g. mutual intermingling) in dual culture are expected to occupy separate nutritional niches, as species utilizing different nutritional sources pose less of a threat to each other than those assimilating the same sources, therefore making the evolution of competitive strategies between these species less likely.

The type of fungal species had a large impact on niche size. On average *F. poae* had the largest niche size of the fungi tested. This was regardless of environmental factors or whether 95 or the key 18 wheat grain carbon sources were considered. The smallest average niche sizes overall were for *F. graminearum* when considering 95 carbon sources, and *M. nivale* var. *majus* when only considering the key 18 carbon sources. The impact of environmental factors on niche sizes was not uniform between species. For instance, changes in niche sizes for *F. culmorum* due to  $a_w$  levels are small compared to *F. graminearum* and *M. nivale* var. *majus* which are subject to large reductions in niche size when  $a_w$  is lowered to 0.93  $a_w$ . Previous studies also noted that species often had a significant effect on niche size and that interaction with  $a_w$  and temperatures did not produce uniform changes (Marin *et al.*, 1998c; Lee & Magan, 1999b).



Niche size for the majority of the fungi increased as  $a_w$  level was increased. Temperature elicited a smaller effect on niche size than  $a_w$ . When 95 carbon sources were considered, incubation at 15 °C generally caused a slight reduction in niche size from those at 25 °C. These temperature and  $a_w$  effects on niche size are similar to those in previous studies (Marin *et al.*, 1998c; Lee & Magan, 1999b). However, when only the 18 key wheat grain carbon sources were considered, the temperature effects on niche size were reversed. This effect was not seen in a previous study that used Biolog plates and compared niche size determined from large number of carbon sources and 18 key maize carbon sources (Lee & Magan, 1999b).

The niche maps presented in this thesis allow the relationships between one species and the target pathogen organism - in this case, *F. culmorum* - to be concisely determined. The niche maps clearly identified *A. tenuissima*, *C. herbarum* and *M. nivale* var. *majus* as occupying separate niches to *F. culmorum* at both 15 and 25 °C for 95 carbon sources assays at 0.995  $a_w$ . However, when only the NOI results for the 18 key wheat carbon sources are considered, all of the fungi assayed were able to exist within the target niche. This highlights the importance of tailoring the carbon sources used in the study to those of the substrate of interest. This view is further supported by Lee & Magan (1999b) who stated that “the spectrum of carbon sources employed in NOI studies should be directly relevant to the habitat of a fungal community to obtain information on the competitiveness of an individual species.”

#### v). *Enzyme profiles as indicators of dominance for Fusarium species*

The carbon source utilization assays and interaction studies (see previous sections above) identified fungi which are most likely to react in a hostile way (exclusion and inhibition interactions) with each other and the way in which these interactions manifest themselves. However, these studies do not fully explain why one fungus may be more dominant over a particular niche than another fungus. This enzyme profile study determined the impact of  $a_w$  and temperature on the activity of hydrolytic enzymes produced by the fungi studied in the NOI and interaction experiments. Previous studies have shown that hydrolytic enzymes can play an important role in the colonisation and growth of a fungus on a substrate (Marin *et al.*, 1998b; Keshri & Magan, 2000) and therefore should also be important factors in fungal interactions. This theory has been proposed in literature but comparisons between enzyme profile



results and an interaction study have not previously been made (Rayner *et al.*, 1995; Marin *et al.*, 1998b).

The enzyme activity profiles generated by the current study, in combination with the findings of the interaction and carbon source utilization studies form part of a comprehensive view on how and to some extent, why, *F. culmorum* competes with other wheat grain spoilage species.

$\alpha$ -D-galactosidase,  $\beta$ -D-glucosidase,  $\beta$ -D-xylosidase and *N*-acetyl- $\beta$ -D-glucosaminidase were produced readily by all of the fungi.  $\beta$ -D-fucosidase,  $\alpha$ -D-mannosidase and *N*-acetyl- $\alpha$ -D-glucosaminidase activity was generally not present, or was low. The exceptions to this were total activity of  $\beta$ -D-fucosidase by *A. tenuissima* and total activity of  $\alpha$ -D-mannosidase by *F. culmorum*, *F. graminearum*, and *P. verrucosum*.  $\beta$ -D-glucosidase produced the highest total activity of the seven enzymes in all of the fungi tested. The fungus that was able to generate the highest average total activity was *P. verrucosum*. However,  $\alpha$ -D-galactosidase almost achieved the same total activity as  $\beta$ -D-glucosidase for *F. culmorum* and *M. nivale* var. *majus* infected grain. Specific activities for  $\beta$ -D-glucosidase were on average higher than any of the other enzymes for all of the fungi except *M. nivale* var. *majus*, where  $\alpha$ -D-galactosidase specific activity was higher.

Previous studies on enzyme production by *F. verticillioides*, and *F. proliferatum* on a variety of substrates give similar results to those data obtained for the fungal species in the current study (Marin *et al.*, 1998b; Keshri & Magan, 2000; Reynoso *et al.*, 2002). These studies all found  $\alpha$ -D-galactosidase,  $\beta$ -D-glucosidase, and *N*-acetyl- $\beta$ -D-glucosaminidase to be produced by the *Fusaria*. However, in contrast with the findings of the current study, no  $\beta$ -D-xylosidase activity was detected.

Temperature,  $a_w$  and time had an effect on both the total and specific enzyme activities. Temperature generally caused a small increase in both total and specific activities when increased from 15 to 25 °C. Longer incubation periods also resulted in an increase in enzyme activities. However, this was markedly greater for total activity than specific activity. The  $a_w$  level of the irradiated wheat grain had a large effect on

both types of enzyme activity. Generally, enzyme activity was seen to increase as  $a_w$  was increased. Previous studies noted similar effects of  $a_w$  and time on enzyme production. However, none of these studies considered the impact of temperature (Marin *et al.*, 1998b; Keshri & Magan, 2000; Reynoso *et al.*, 2002). In the current study all of the factors and their interactions were significant apart from interactions with temperature.

A particular point of interest is that all the *Fusaria* produced higher *N*-acetyl- $\beta$ -D-glucosaminidase mean specific activity than any of the other fungi. This may indicate that *N*-acetyl- $\beta$ -D-glucosaminidase plays an important role in the colonisation strategies of these species and may have been partly responsible for the dominant interactions the *Fusarium* species were able to produce in the interaction study. *N*-acetyl- $\beta$ -D-glucosaminidase is an important extracellular and cell wall component of some fungi (Brunner *et al.*, 2003). It is required for the successful degradation of N-glycosylproteins, important components of many plants and fungi (Karamanos *et al.*, 1995). In addition to the nutritional benefit that *N*-acetyl- $\beta$ -D-glucosaminidase has, it is also linked to the production of chitinase, an important biocontrol enzyme implicated in activity of biocontrol species (Brunner *et al.*, 2003).

#### **4.4 THE EFFECT OF FUNGICIDES ON *F. CULMORUM* GROWTH, MYCOTOXIN PRODUCTION AND INTERSPECIFIC INTERACTIONS**

The literature review discussed how fungal spoilage in grain is currently controlled with fungicides and effective storage regimes. The profile and interaction experiments in this study addressed the effects on various aspects of *F. culmorum* physiology of temperature,  $a_w$  and interspecific interactions similar to those experienced during the drying and storage of wheat grain. This study also investigated the effect that three common cereal fungicides (azoxystrobin, epoxiconazole and propiconazole) have on *F. culmorum* growth, interactions with other species and mycotoxin production.

Azoxystrobin is an agrochemical fungicide belonging to the strobilurins group. This group of fungicides inhibits mitochondrial respiration by blocking electron transport.



This results in growth reduction due to the decrease in aerobic metabolism. Propioconazole and epoxiconazole are triazole-based fungicides which inhibit fungi using a different mechanism of action to azoxystrobin. The antifungal effects of this group of fungicides are mainly attributed to inhibition of ergosterol biosynthesis (Hewitt, 1998). The effects of ergosterol biosynthesis inhibition in fungi cause morphological malformations, prolific branching and irregularities in cell wall thickness. Fungal growth rate and metabolism are not immediately affected, requiring the depletion of intercellular stores of sterol first (Siegel, 1981).

*i). Effect of fungicides on growth*

Comparisons with previous fungicide studies are possible because although many of these looked at the effect of fungicides in the field (Homdork *et al.*, 2000a; Edwards *et al.*, 2001; Simpson *et al.*, 2001; Gullino *et al.*, 2002), the current study covers the  $a_w$  ranges and corresponding moisture content levels present post anthesis in wheat grain. This period of grain development is of key importance as, 10-20 days post anthesis the grain is in the milky ripe stage of development and has a moisture content of approximately 70% ( $a_w = 1.00$ ). It is at this time that the grain is most susceptible to fungal infection (Magan & Lacey, 1985). As the grain ripens, moisture levels drop to around 20% ( $a_w = 0.92$ ). Therefore, the  $a_w$  ranges in the current study are very relevant to fungal infection and colonisation occurring on wheat grain in the field.

Azoxystrobin was unable to effectively control (<50 %) *F. culmorum* growth, although there was a slight reduction in growth rates. Optimal reduction in growth occurred at  $25 \mu\text{g g}^{-1}$ ; increasing the concentration to  $50 \mu\text{g g}^{-1}$  resulted in either no significant change in growth rate, or a slight increase. Stimulation of growth occurred in all conditions with azoxystrobin concentrations of  $1 \mu\text{g g}^{-1}$ . In a previous study azoxystrobin was also seen to be ineffective on wheat agar, and to cause stimulation of growth under some conditions. When applied to irradiated wheat grain azoxystrobin was also ineffective in controlling mycelial growth of *F. culmorum*. However, no stimulation of fungal growth occurred, as was seen when azoxystrobin was used on a wheat agar substrate (Magan *et al.*, 2002). Azoxystrobin was also found to be ineffective at reducing the percentage of *F. culmorum* in winter wheat field trials (Simpson *et al.*, 2001).



Both propiconazole and epoxiconazole were able to cause large reductions in *F. culmorum* growth rate. Growth rate was generally reduced as fungicide concentration increased. The exception to this was at a concentration of  $1 \mu\text{g g}^{-1}$  where no significant reduction in growth was observed at 0.955  $a_w$ . LD<sub>50</sub> values for propiconazole and epoxiconazole were  $5 \mu\text{g g}^{-1}$  in all of the  $a_w$  and temperature treatment conditions, except at 0.98  $a_w$  with epoxiconazole. This is similar to results found on agar and grain in a previous study (Magan *et al.*, 2002).

### ii). Index of dominance and fungicides

The addition of fungicides often had no effect on the outcome of fungal interactions. However, in conditions where fungicides did affect interaction results, it normally caused interactions to change in favour of *F. culmorum*. For instance, the mutual inhibition interactions between *P. verrucosum* and *F. culmorum* were changed to dominance on contact with *F. culmorum*, when the media was supplemented with propiconazole and epoxiconazole. It could have been due to similar changes in interactions between FEB pathogens and fungicides that resulted in *F. culmorum* dominance of the FEB complex in previous studies (Jennings *et al.*, 2000; Simpson *et al.*, 2001).

### iii). Effect of interactions and sub-optimal levels of fungicides on growth

The addition of sub-optimal levels of fungicide had a significant effect on *F. culmorum* growth rates when it was grown in dual culture with competing species. Azoxystrobin and epoxiconazole generally caused a significant stimulation in *F. culmorum* growth when in dual culture compared to growth rates determined from plates untreated with a fungicide. This is in agreement with previous studies that also found azoxystrobin to be ineffective against *F. culmorum* (Jennings *et al.*, 2000; Edwards *et al.*, 2001; Simpson *et al.*, 2001; Magan *et al.*, 2002; Pirgozliev *et al.*, 2002). Propiconazole treatment had the opposite effect on growth to the other fungicides tested, reducing *F. culmorum* growth in dual culture compared to that of untreated controls. Propiconazole has also been found to be effective against *Fusarium* species in previous experiments. For instance, in a study on three systemic fungicides on *F. graminearum* growth and DON production on wheat ears, propiconazole resulted in a 34-79 % decrease of infection and DON production (Boyacioglu *et al.*, 1992). In a more recent study the fungicides prochloraz,



propioconazole, epoxiconazole, tebuconazole and azoxystrobin were shown to be able to reduce growth of *F. graminearum* across the  $a_w$  range of 0.95 – 0.99 $a_w$  (Ramirez *et al.*, 2003).

The effect of some interactions on *F. culmorum* growth was reversed. For instance, interactions between *F. culmorum* and *F. graminearum* caused a reduction in *F. culmorum* growth. Under the same conditions but with a treatment of 0.5  $\mu\text{g}^{-1}$  azoxystrobin growth rate was increased. No previous study has attempted to determine the effect of fungicides on growth of competing fungal species. However, the data in the current study can help explain the reasons why in previous studies the applications of fungicides has sometimes resulted in an increased severity of FEB or *F. culmorum* levels (D'Mello *et al.*, 1998; Jennings *et al.*, 2000; Simpson *et al.*, 2001).

*iv). Effects of interactions and sub-optimal fungicide concentrations on DON and NIV levels*

The addition of the fungicides had a significant effect on mycotoxin production by *F. culmorum* when in the presence of a competing fungus. The fungicides also had a significant effect on the DON and NIV levels in the homogenous controls. For instance, on the agar substrate DON levels were completely inhibited by azoxystrobin, whereas with epoxiconazole and propiconazole DON levels were stimulated. There was no discernable pattern to mycotoxin stimulation or suppression in dual cultures treated with fungicide, but all of the factors did elicit a statistically significant effect.

Field trials carried out on ripening wheat ears have also noted the variable effects that fungicides can have on controlling DON levels. It was found that treatment of FEB by azoxystrobin caused a 40% increase in DON over levels in untreated samples, even though the levels of *Fusarium* were reduced by 20% by the application of azoxystrobin. When tebuconazole was used to control the FEB infection DON levels were 80% lower than in the control (Jennings *et al.*, 2000; Simpson *et al.*, 2001). This is in contrast to the current study where no significant stimulation of toxin production was seen by azoxystrobin. This may be due to a change in the pattern of fungi present in the field. In a recent study the fungicides prochloraz, propiconazole, epoxiconazole, tebuconazole and azoxystrobin in combination with water and

temperature factors were shown to have a complex interaction with DON production by *F. graminearum*. Under some conditions stimulation of DON was seen, while under others, a reduction was observed (Ramirez *et al.*, 2003)

Previously a study has shown that the magnitude of growth inhibition by a fungicide is not necessarily related to its effect on mycotoxin production (D'Mello *et al.*, 1998). This is supported by the current study that also found no correlation between a fungicides effect on growth and its effect on mycotoxin production.

It is possible that fungicides act as an additional stress factor, causing stimulation of mycotoxin synthesis as a defence response (Magan *et al.*, 2002). Studies have shown that metaconazole or tebuconazole are not only able to inhibit growth but also cause morphological and cytochemical alterations of the hyphae when 20  $\mu\text{g ml}^{-1}$  of the fungicides was added to the media (Kang *et al.*, 2001a; Kang *et al.*, 2001b). Kang *et al.* (2001b) also showed via immunogold labelling that DON was accumulated in the cell walls, cytoplasm, mitochondria and vacuoles of the hyphae in the control and the fungicide treatment. However, there was significantly less DON in these cellular components in the hyphae exposed to the fungicides.

#### **4.5 CONTROL OF *F. CULMORUM*, *F. GRAMINEARUM* AND *F. POAE* COLONISATION AND ASSOCIATED MYCOTOXIN PRODUCTION USING ESSENTIAL OILS AND ANTIOXIDANTS**

The current study has shown that only a few of the essential oils and antioxidants tested were effective in controlling growth of the three *Fusarium* species tested. The efficacy of these treatments was however affected by changes in environmental stress caused by altering the  $a_w$  and temperature levels. Of particular importance was the fact that while low concentrations (50-100 ppm) were effective *in vitro*, much higher concentrations (500 ppm) were required to control growth of *Fusaria in situ* on wheat grain. This suggests that care is needed in extrapolating from *in vitro* to *in situ* studies with such antimicrobial compounds, especially when considering durable crops.



However, the results obtained from the *in situ* study show that the best essential oils/antioxidants can significantly reduce growth as well as mycotoxin production across a range of conditions similar to those encountered in grain during drying. Therefore, it is possible that the application of essential oils/antioxidants could provide an important tool in the control of *F. culmorum*, *F. graminearum* and *F. poae* and their associated toxins during drying - a critical control point in the cereal production chain.

In some cases stimulation in growth or mycotoxin production was observed. It was noticeable that with regard to growth, the biomass production may not have increased relative to the growth rate. However, the morphology of the colonies did change under different conditions. For example, *F. culmorum* colonies at 0.995  $a_w$  and 25 °C consisted of dense mycelial growth with characteristic dark red/brown pigmentation. The colonies produced at 0.985  $a_w$  were formed of diffuse pale mycelium with much less floccose growth.

Studies conducted to determine the antimicrobial effect of essential oil active components have been carried out. However, these studies have produced conflicting results. The inhibition of *Aspergillus parasiticus* was found to be higher for the components of carrot seed oil (limonene and terpinene) than for the complete oil (Batt *et al.*, 1983). In contrast the oils derived from orange or lemon peel were more effective at controlling growth and aflatoxin production than was d-limonene, the main constituent of the two peel oils (Alderman & Marth, 1976). Paster *et al.* (1995) found that thyme essential oil was more inhibitory than the individual components. It was suggested that essential oil activity resulted mainly from synergistic or cumulative effects existing between the components. However, more information on the mode of action employed by oil is required to identify such possible synergism.

The modes of action of essential oils are largely unknown. However, there is some information in the literature towards this end. In a study of Egyptian essential oils by Farag *et al.* (1989) it was concluded that there was a relationship between the chemical structure of the main component of an essential oil and its antimicrobial activity. The inhibitory effect of the oils was generally attributed to the presence of an

aromatic nucleus containing a polar functional group. This is a similar structure to that of phenols and chlorophenols, which are widely used in disinfectants. The presence of phenolic OH groups able to form hydrogen bonds with the active sites of target enzymes was thought to increase antimicrobial activity. It was also stressed that the inductive effect of isopropyl groups should also be taken into consideration.

The essential oils used in the current study have been analysed by GC-MS to determine their composition. This information was kindly provided by Prof. P.V. Nielsen, DTU, Denmark. The major component of bay, cinnamon and clove essential oils was eugenol. In a study of over 50 essential oils, eugenol was found to be a major component in many of the oils that exhibited antifungal and antibacterial effects (Lis-Balchin *et al.*, 1998). This study noted that cinnamaldehyde was also an important antifungal component. Cinnamaldehyde and eugenol were also found to inhibit mould growth in earlier studies (Bullerman *et al.*, 1977). Cinnamaldehyde was detected in the cinnamon essential oil used in the current study but it was only present at a low percentage 1.88 %, whereas eugenol constituted 71.92% of the oil.

Of the antioxidants, BHA and propyl paraben were effective inhibitors of mycelial growth and toxin production under some conditions. This agrees with a recent study by Etcheverry *et al.* (2002) who found that BHA and propyl paraben inhibited *Fusarium verticillioides* and *Fusarium proliferatum* growth and production of fumonisin. Subsequent studies also suggested that using low concentrations of the two antioxidants may have a synergistic inhibitory effect on growth of *Fusarium* section *Lisoela* species and on fumonisin production (Reynoso *et al.*, 2002).

The mechanism of action for the parabens which produce inhibition of mycelium in toxigenic fungi is not clear. However, it is known that the effectiveness of the parabens increases with an increase in the chain length of the ester group (Thompson, 1992; Thompson *et al.*, 1993). Khan *et al.* (2001) suggested that propyl paraben and BHA appear to work mainly at the cell membrane eliminating the pH component of the protomotive force and affecting energy transduction and substrate transport. BHA has also been shown to have a direct effect on the mitochondrial electron chain of trypanosomes, thus inhibiting respiration.



Stimulation of growth and toxin production was seen in some of the conditions examined. Water activities of 0.955  $a_w$  and treatment concentrations of 100 ppm produced the most dramatic stimulation. Stimulation or inhibition of growth and mycotoxin production were not always mutually exclusive. For example, growth of *F. culmorum* was significantly inhibited by 500 ppm cinnamon oil at 0.955  $a_w$  / 25 °C, yet toxin production was enhanced. This is similar to the results of Magan *et al.* (2002) who found that sub-optimal levels of fungicides stimulated DON production by *F. culmorum*.

Studies of the effect of propionate preservatives on growth and fumonisin B1 production by *F. verticillioides* and *F. proliferatum* species under various temperatures/ $a_w$  levels on maize grain showed similar results to those presented in this study for essential oils and antioxidants (Marin *et al.*, 1999b). In a more recent study on the effects of several essential oils on *F. proliferatum* growth and fumonisin B1 production, cinnamon was identified as one of the most promising essential oils for use as a control agent. However, as in the present study, cinnamon oil was unable to control mycotoxin levels at 0.95  $a_w$  (Velluti *et al.*, 2003).

#### **4.5.1 Control of *Fusarium* species on naturally contaminated wheat grain using essential oils and antioxidants**

The impact of the essential oils or antioxidants on populations of fungi in naturally contaminated wheat grain was determined by comparing populations of fungi (CFUs) from wheat grain treated with essential oil or antioxidant with those from the untreated controls. The essential oils and antioxidants were able to inhibit total fungal populations by >90 % under the majority of the experimental conditions.

The PP and BHA were the least effective population control treatments and under some conditions caused stimulation in CFU numbers. The largest stimulation (>200 %) in total fungal populations occurred at 0.95  $a_w$ , 15 °C, after 14 days incubation with a BHA treatment. Even one of these less effective treatments had a dramatic effect on visible levels of fungal contamination on the wheat grain.

Mycotoxin analysis of the treated wheat grain after 28 days storage showed that DON and NIV were present in the majority of samples. However, DON was the only toxin present in concentrations greater than 200 ppb. Bay essential oil was able to completely inhibit DON production in all of the  $a_w$  and temperature conditions. Stimulation of DON production only occurred with cinnamon essential oil treatment at 0.97  $a_w$  and 25 °C. Bay essential oil is therefore the most promising control agent for *Fusarium* contaminated wheat grain entering storage.

There has been no comparable study on the effects of essential oils and antioxidants in natural wheat grain systems at present. These agents may offer an effective means for treating grain harvested during wet conditions, to provide more time prior to drying or storage. This critical control point within wheat grain production is currently only controlled by rapid transport of wet grain to a drying facility (Aldrick, 1996; Aldrick & Knight, 2000).

#### **4.5.2 Cost benefit analysis**

One of the main concerns for the uptake of new antifungals is the cost. Bay essential oil which was the most effective oil of those tested at controlling fungal contamination and DON/NIV production costs £224.60 per Kg. Therefore, to treat 1 ton (1000 Kg) of grain with a 200  $\mu\text{g g}^{-1}$  concentration of bay oil would cost £40. This treatment has been shown to reduce fungal populations and DON/NIV levels by >90% in 100 g grain samples of naturally contaminated wheat grain. There are currently no commercially available post-harvest fungicides with which to compare the costs.



# **CHAPTER 5**

## **CONCLUSIONS AND FURTHER WORK**

## 5.1 CONCLUSIONS

The following conclusions can be drawn based on the experimental work of this study.

1. Environmental variables ( $a_w$  and temperature) and their interactions had a significant effect on growth, DON/NIV productions and interspecific interactions
2. Wet conditions ( $0.995 a_w - 0.98 a_w$ ) and high temperatures ( $25\text{ }^{\circ}\text{C}$ ) produced the highest growth rates in all of the *Fusarium* species studied.
3. Two dimensional profiles for *F. culmorum* and *F. graminearum* for the first time showed that:- a). DON and NIV production by *F. culmorum* and *F. graminearum* was highest at  $a_w$  levels comparable with grain during a wet harvest. b). The optimum conditions for mycotoxin production were not always those for optimum growth.
4. Interspecific interactions had significant effects on *F. culmorum* growth, mycotoxin production and dominance in the wheat grain niche. Interactions were able to cause reduction or stimulation of growth and mycotoxin production, but the interaction effects often depended on the environmental conditions.
5. The enzyme *N*-acetyl- $\beta$ -D-glucosaminidase was produced in significantly higher quantities by the *Fusarium* species than the other fungal species tested. *Fusarium* species were also the most dominant in the interaction experiments, suggesting a possible link between *N*-acetyl- $\beta$ -D-glucosaminidase production and dominance. However, further work is required to clarify this point.
6. Sub-optimal levels of fungicides commonly used to control *Fusarium* ear blight caused stimulation of growth and mycotoxin production under some conditions. Presence of these fungicides can also increase *F. culmorum*'s dominance over other spoilage fungi both *in vitro* and *in situ*.



7. Butylated hydroxyanisole, propyl paraben and the essential oils clove, cinnamon and bay were able to control (>90 %) the growth of *F. culmorum*, *F. graminearum* and *F. poae* at 500 ppm *in vitro* on wheat agar and *in situ* on irradiated wheat grain. DON and NIV production was often also suppressed under these conditions.
8. Bay essential oil is the most effective of the antioxidants and essential oils tested at reducing fungal growth and DON and NIV production in tests on naturally contaminated wheat grain.

## 5.2 FURTHER WORK

Possible further works based on the findings of this study are:

1. The use of computer modelling (i.e. neural networks) to predict *F. culmorum* infection and mycotoxin production based on data for  $a_w$ , temperature and presence of other fungal species on wheat grain. This would prove a useful risk assessment tool.
2. Study the interspecific interactions between wheat grain contaminant fungi and *Fusarium* species mutants unable to produce *N*-acetyl- $\beta$ -D-glucosaminidase, to clarify this enzyme's role in *Fusarium* interactions.
3. Investigate economics of bay essential oil application for controlling *Fusarium* infection and mycotoxins on other cereals, and whether bay essential oil can be applied as a fumigant in grain stores.
4. Use of green fluorescent protein marked strains to examine infection and colonisation of grain.

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# APPENDIX I

## **I.I HYDROLYTIC ENZYME PROFILES FOR GRAIN SPOILAGE FUNGI**



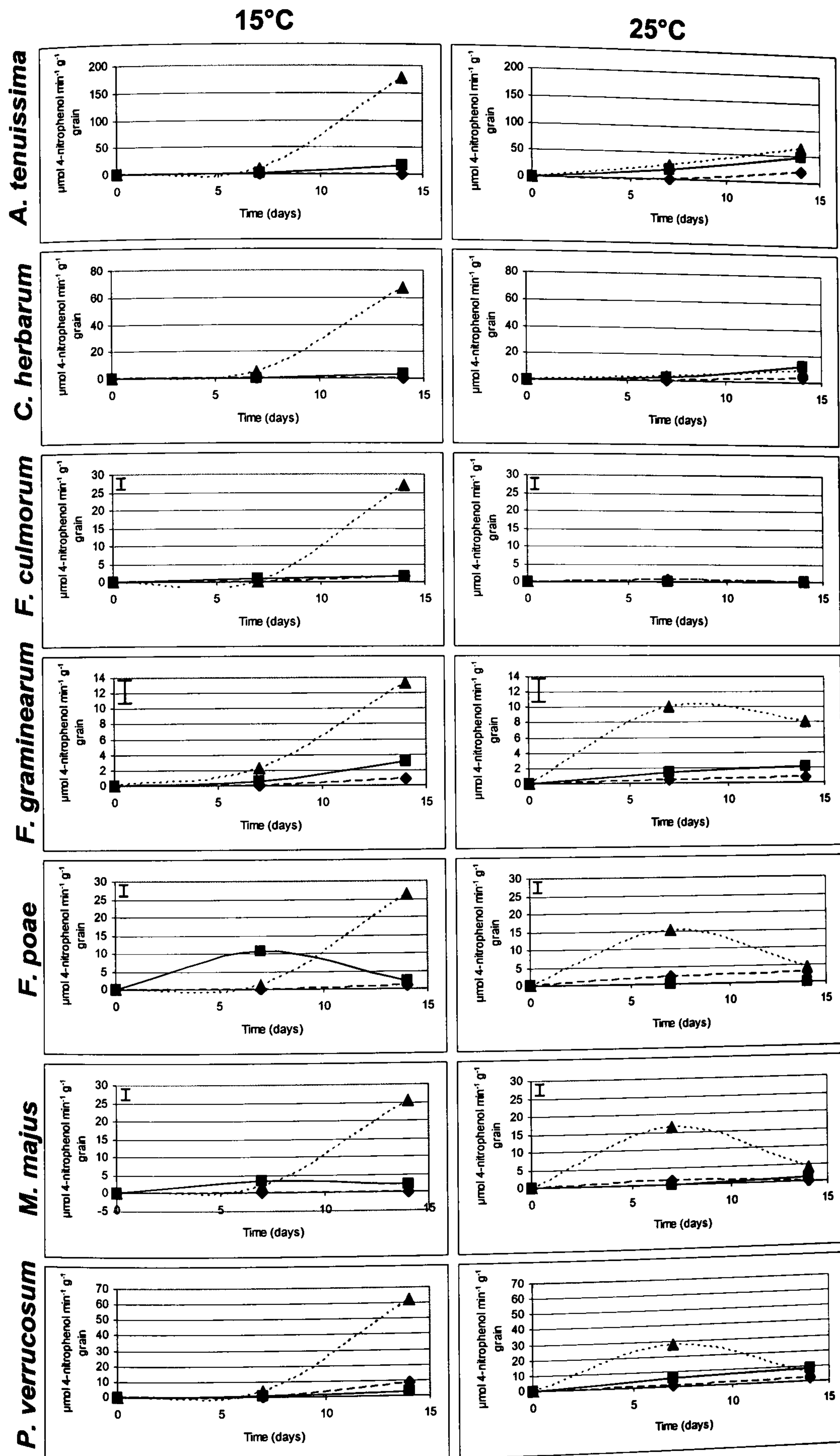


Figure I.1 Total  $\beta$ -D-fucosidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to  $\blacktriangle$  0.99  $a_w$ ,  $\blacksquare$  0.95  $a_w$  and  $\blacklozenge$  0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker.

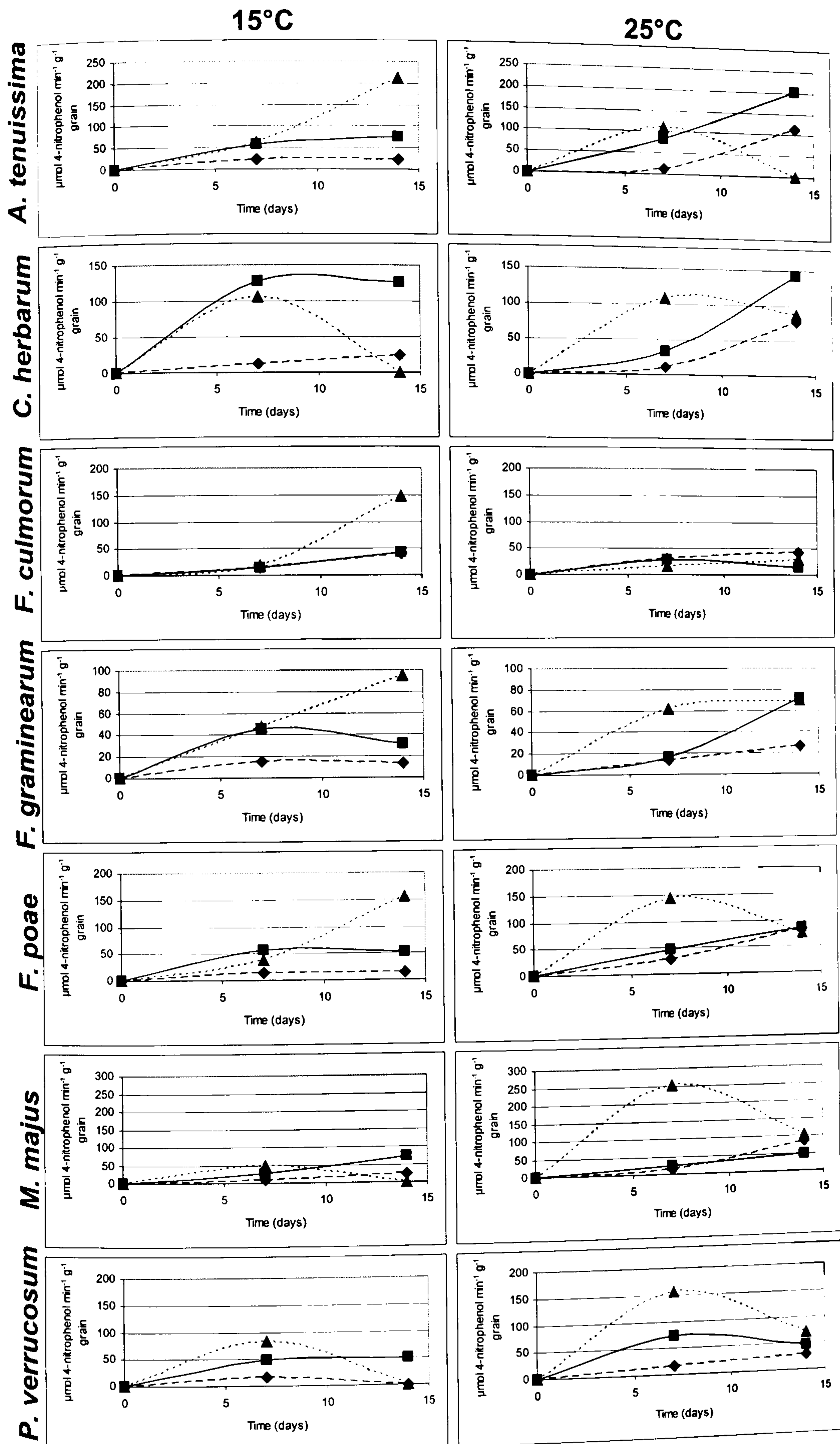


Figure I.2 Total  $\alpha$ -D-galactosidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to  $\blacktriangle$  0.99  $a_w$ ,  $\blacksquare$  0.95  $a_w$  and  $\blacklozenge$  0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker.



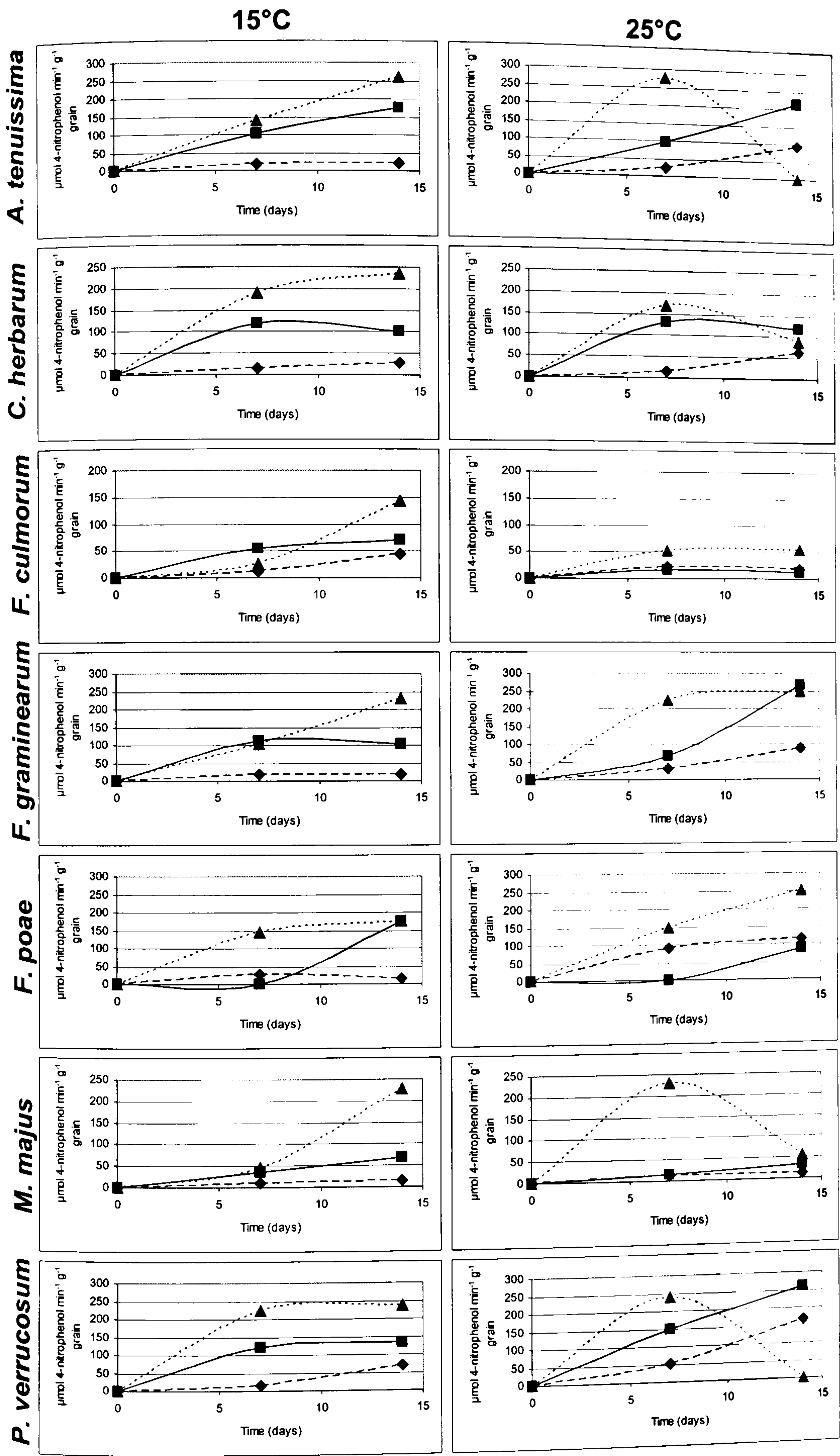


Figure I.3 Total  $\beta$ -D-glucosidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to  $\blacktriangle$  0.99  $a_w$ ,  $\blacksquare$  0.95  $a_w$  and  $\blacklozenge$  0.93  $a_w$ . Incubated at 15  $^{\circ}\text{C}$  or 25  $^{\circ}\text{C}$ . LSD shown where larger than marker

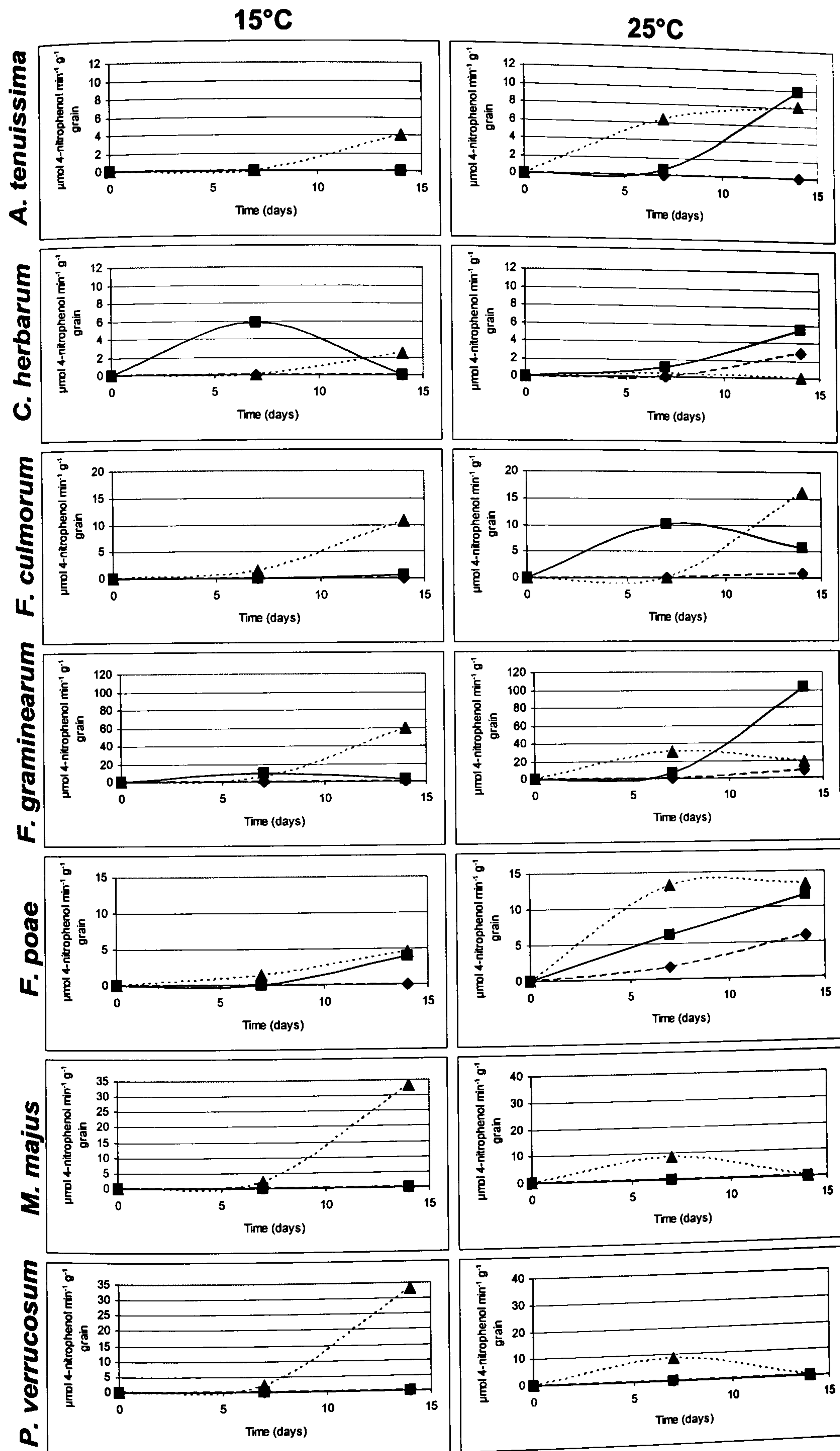


Figure I.4 Total  $\alpha$ -D-mannosidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to  $\blacktriangle$  0.99  $a_w$ ,  $\blacksquare$  0.95  $a_w$  and  $\blacklozenge$  0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker



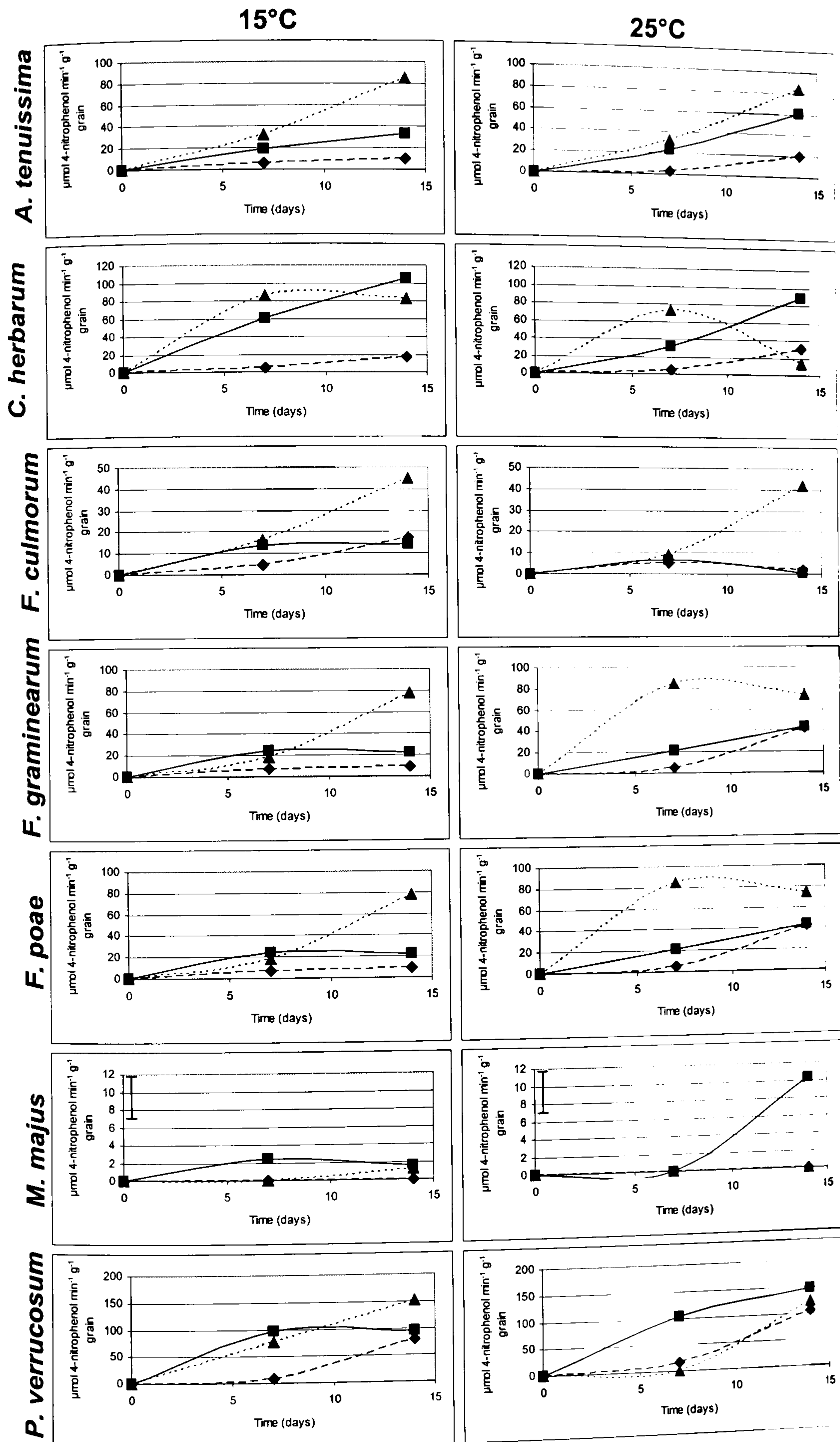


Figure I.5 Total  $\beta$ -D-xylosidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to ▲ 0.99  $a_w$ , ■ 0.95  $a_w$  and ♦ 0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker

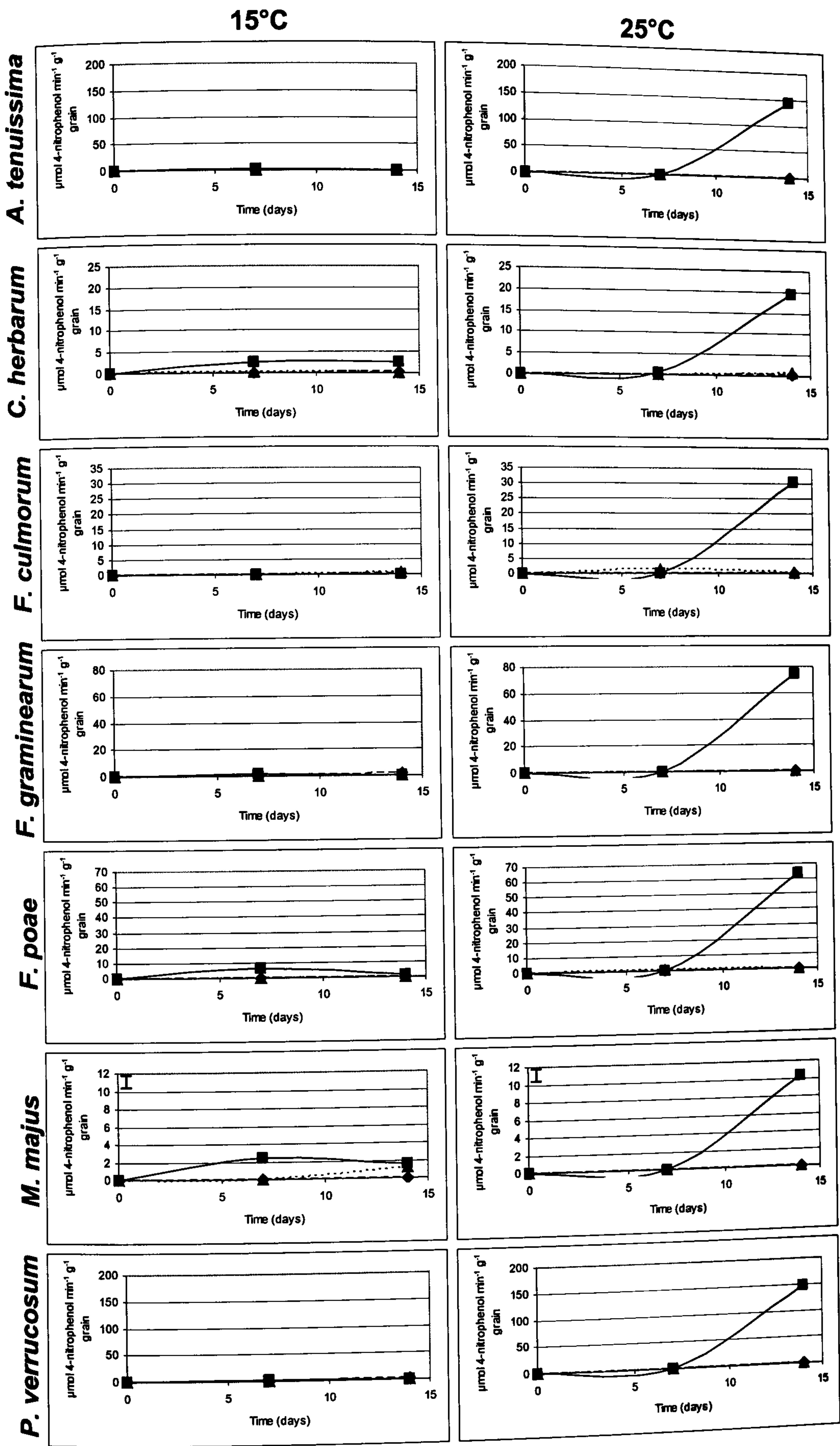


Figure I.6 Total *N*-acetyl- $\alpha$ -D-glucosaminidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to  $\blacktriangle$  0.99  $a_w$ ,  $\blacksquare$  0.95  $a_w$  and  $\blacklozenge$  0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker



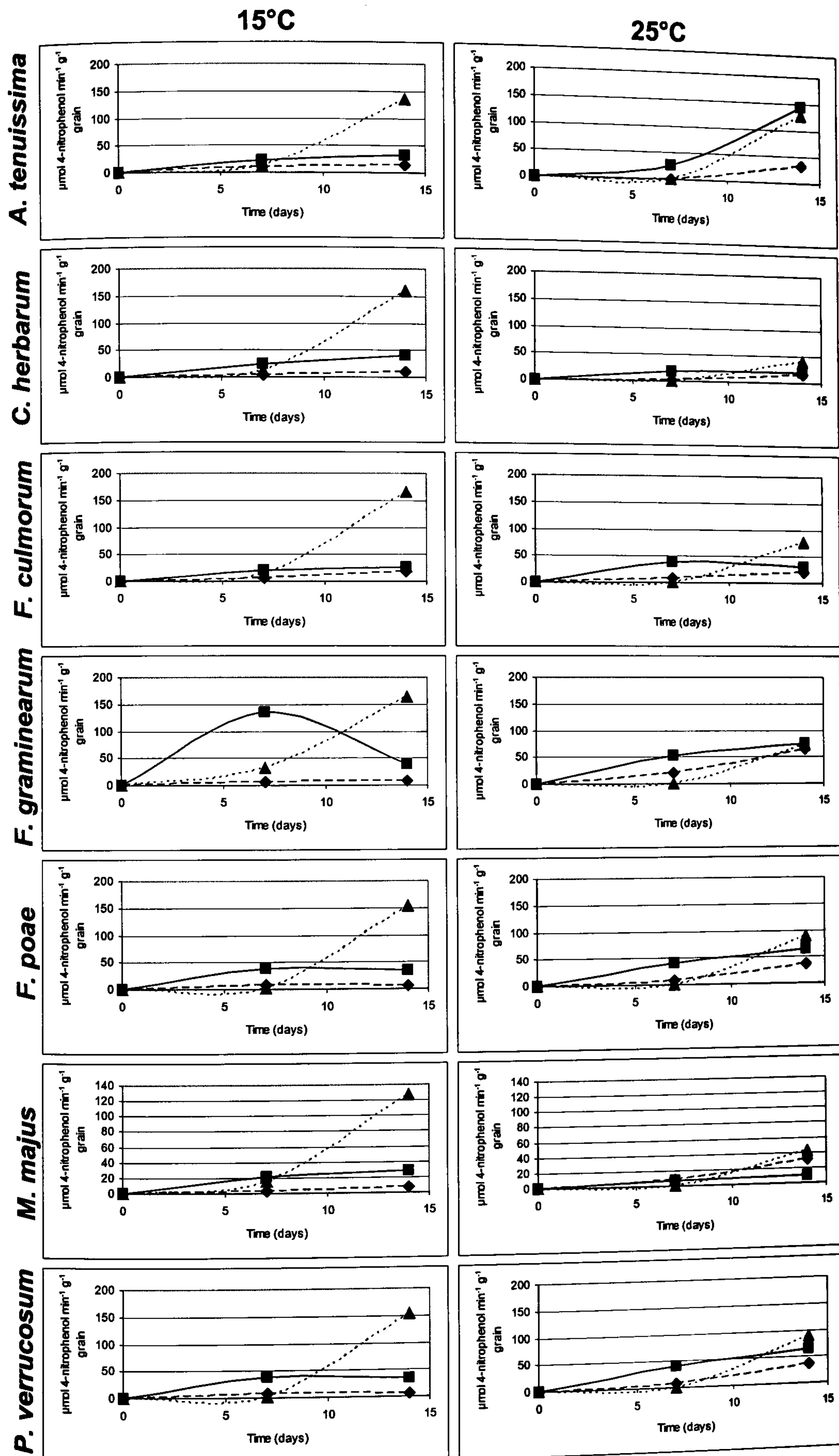


Figure I.7 Total *N*-acetyl- $\beta$ -D-glucosaminidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to ▲ 0.99  $a_w$ , ■ 0.95  $a_w$  and ◆ 0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker

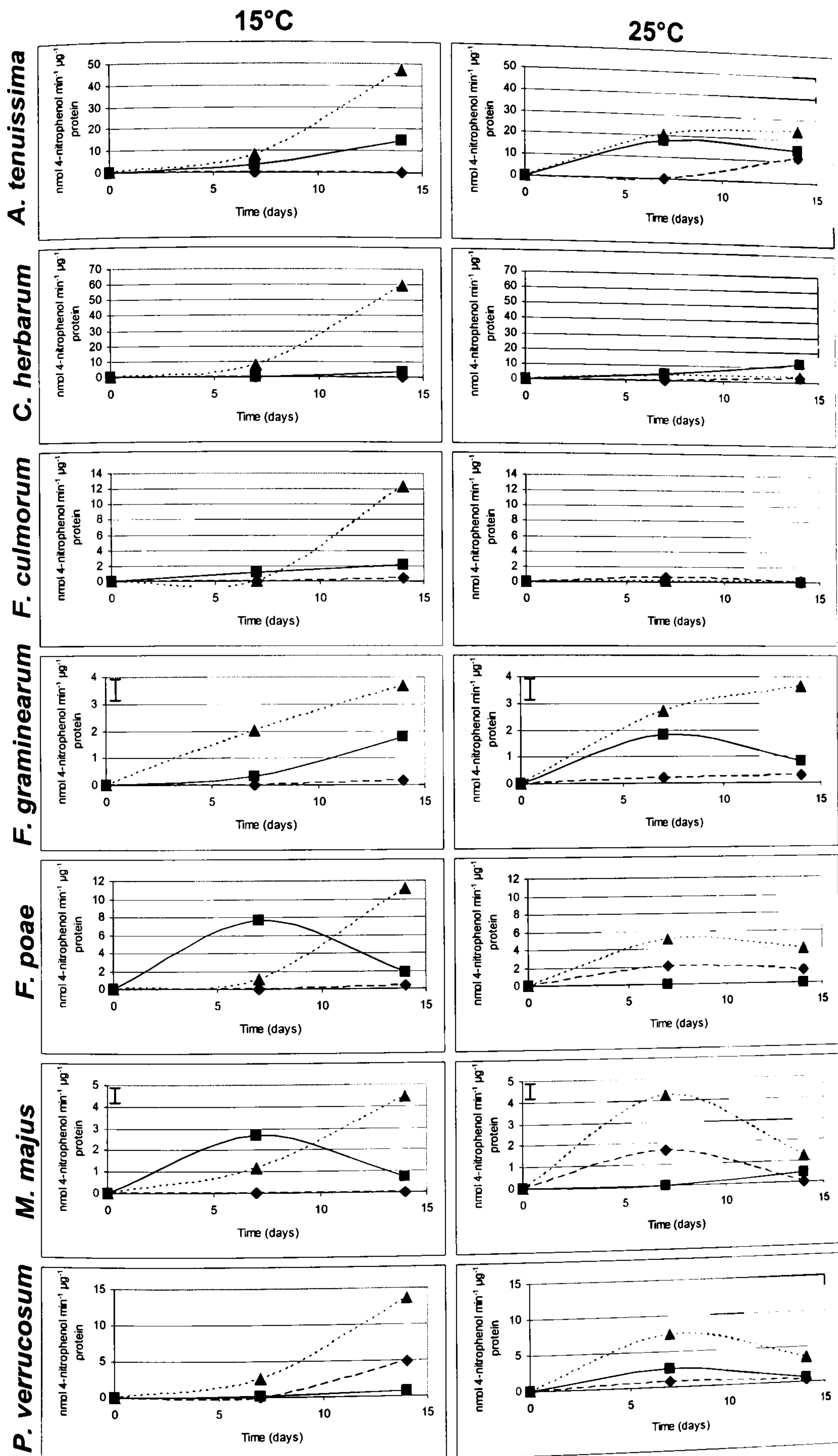


Figure I.8 Specific  $\beta$ -D-fucosidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to ▲ 0.99 a<sub>w</sub>, ■ 0.95 a<sub>w</sub> and ◆ 0.93 a<sub>w</sub>. Incubated at 15 °C or 25 °C. LSD shown where larger than marker



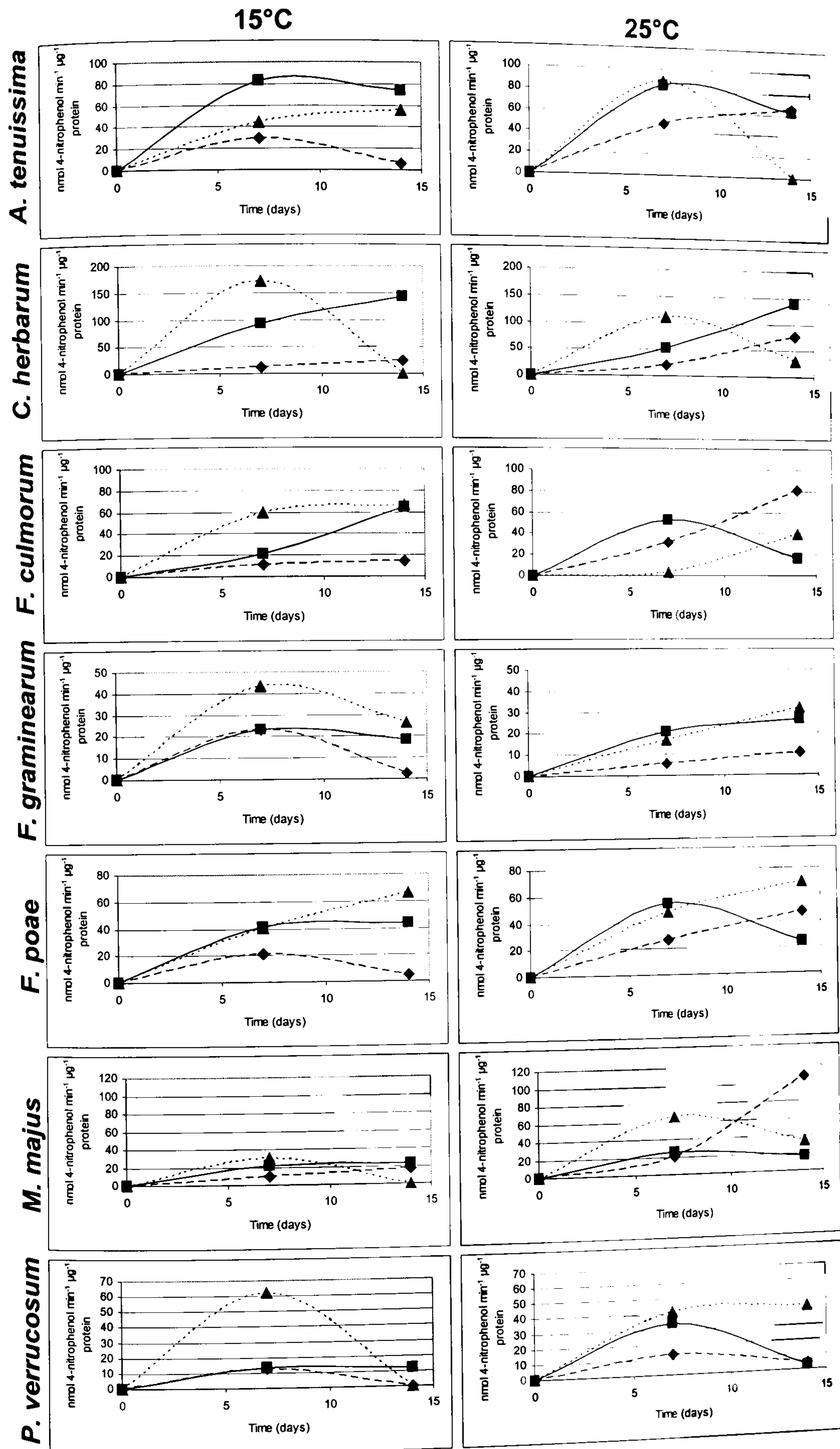


Figure I.9 Specific  $\alpha$ -D-galactosidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to ▲ 0.99 a<sub>w</sub>, ■ 0.95 a<sub>w</sub> and ◆ 0.93 a<sub>w</sub>. Incubated at 15 °C or 25 °C. LSD shown where larger than marker

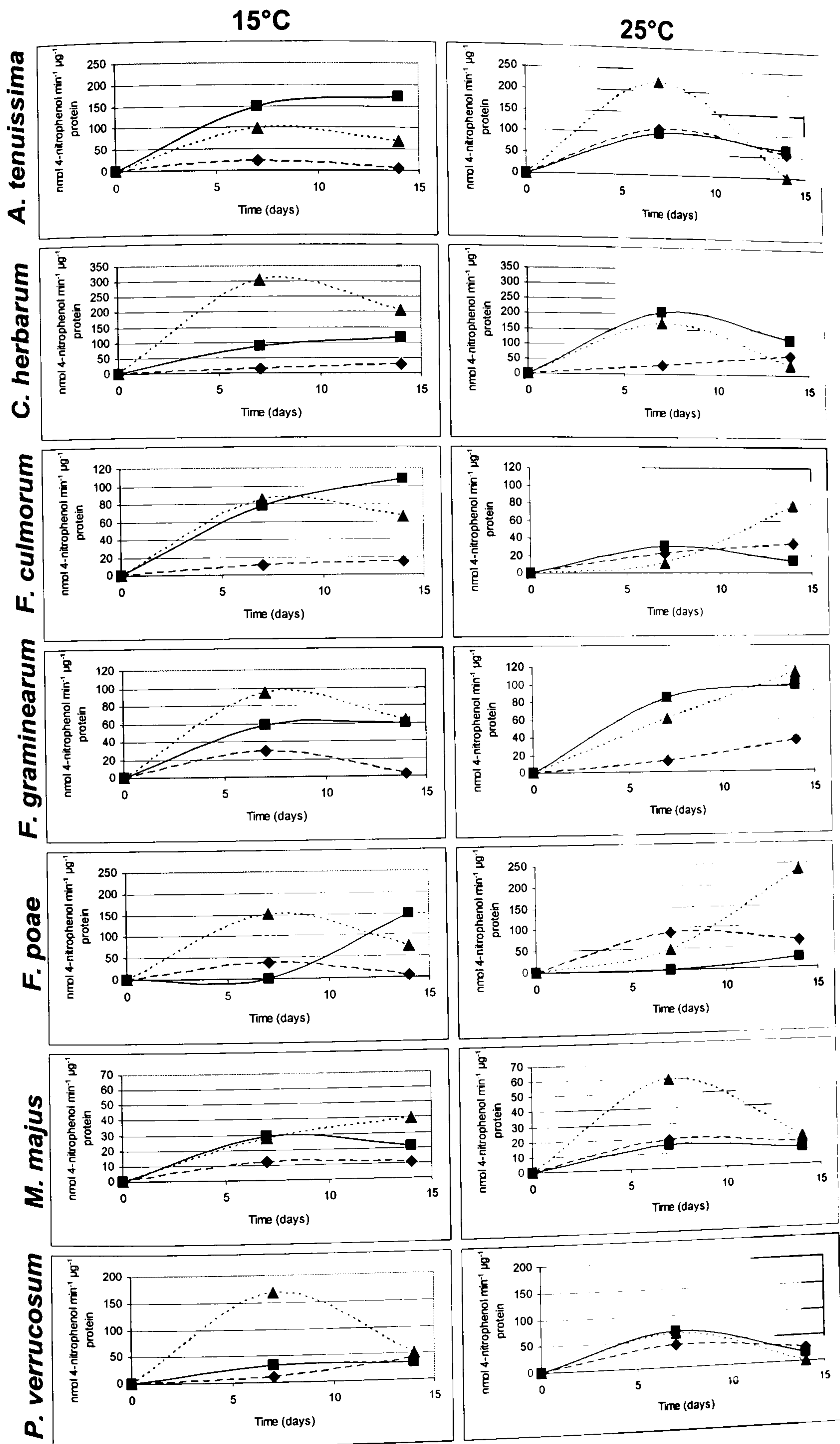


Figure I.10 Specific  $\beta$ -D-glucosidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to  $\blacktriangle$  0.99  $a_w$ ,  $\blacksquare$  0.95  $a_w$  and  $\blacklozenge$  0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker



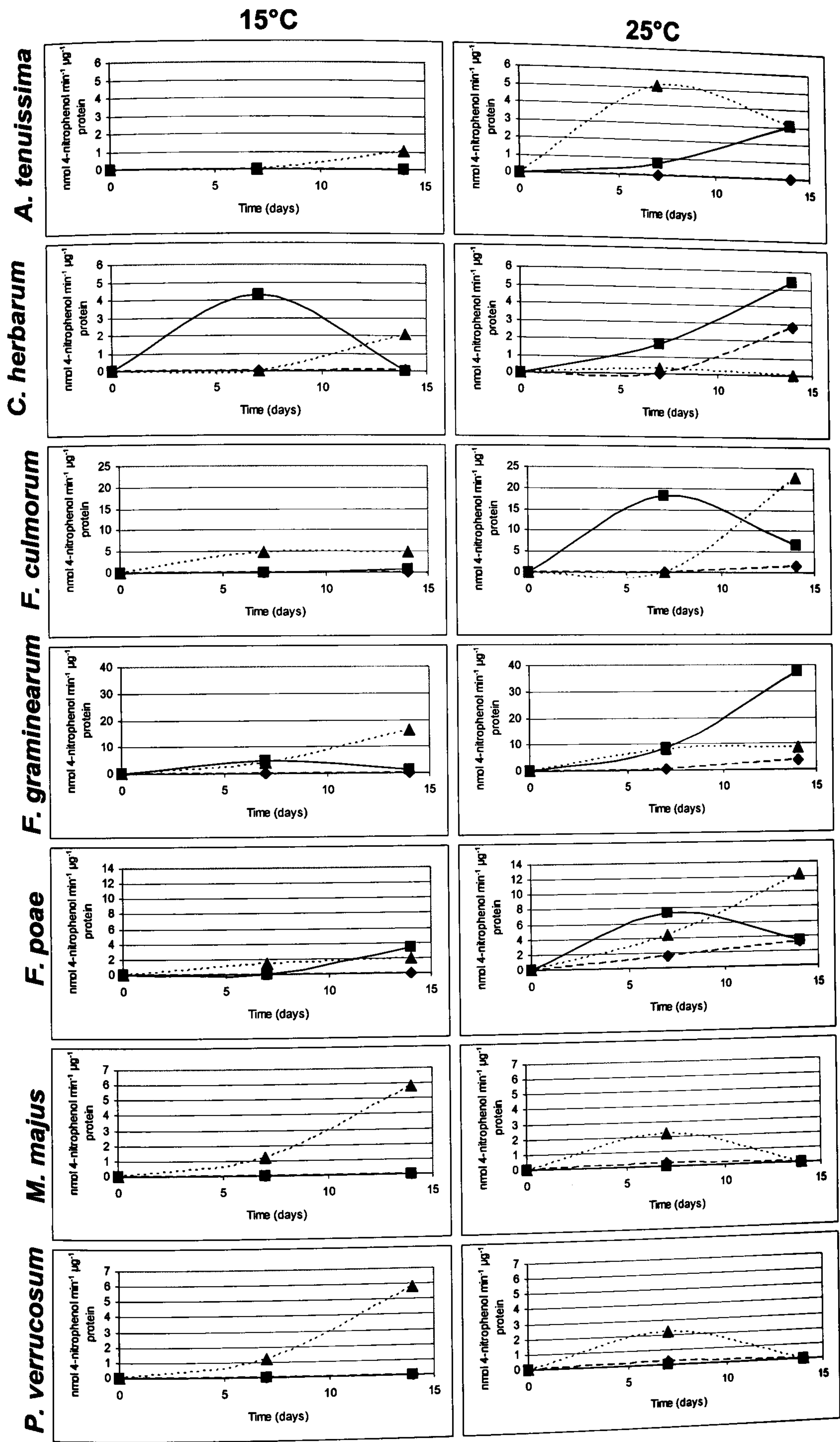


Figure I.11 Specific  $\alpha$ -D-mannosidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to  $\blacktriangle$  0.99  $a_w$ ,  $\blacksquare$  0.95  $a_w$  and  $\blacklozenge$  0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker

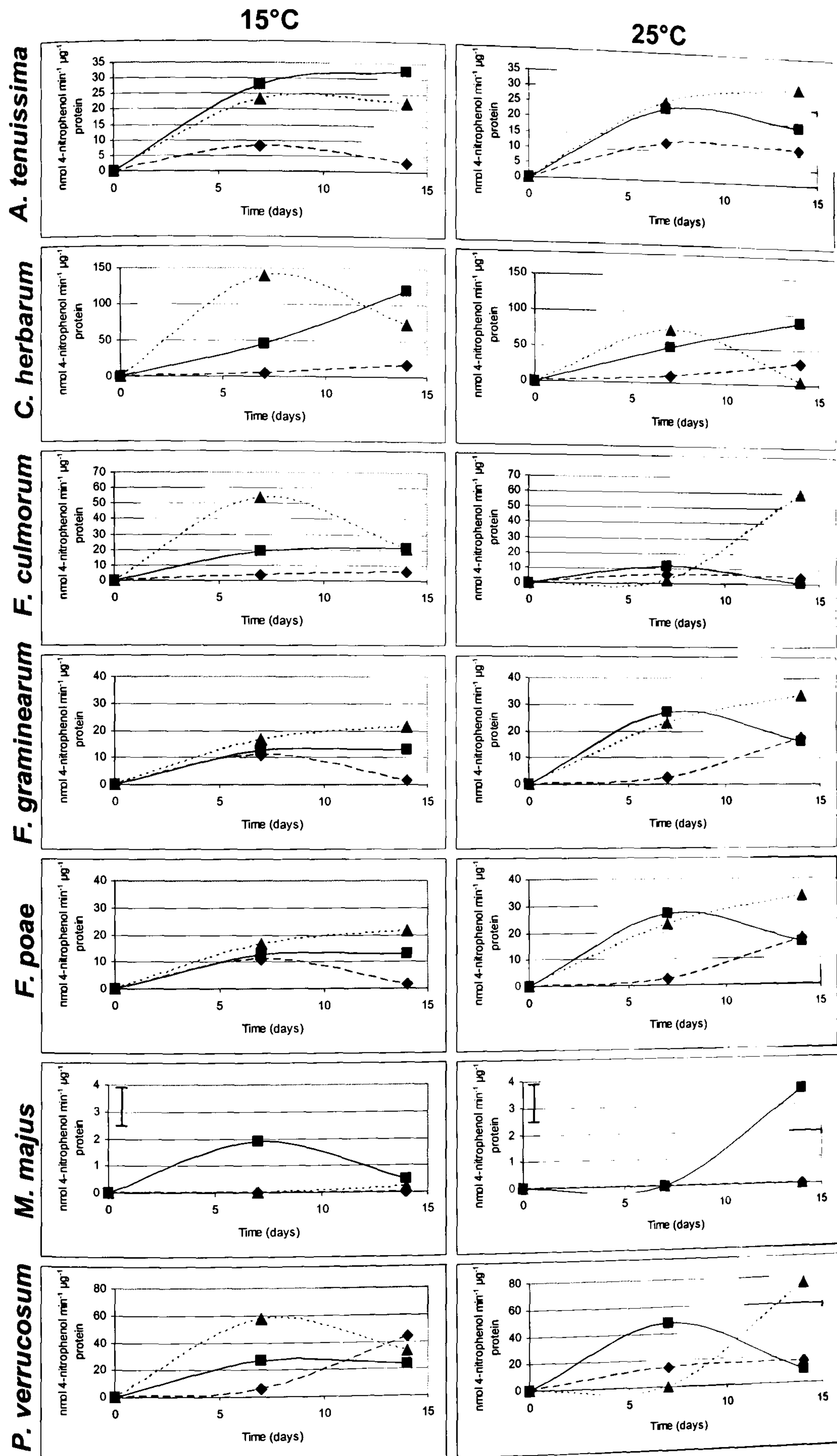


Figure I.12 Specific  $\beta$ -D-xylosidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to  $\blacktriangle$  0.99  $a_w$ ,  $\blacksquare$  0.95  $a_w$  and  $\blacklozenge$  0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker



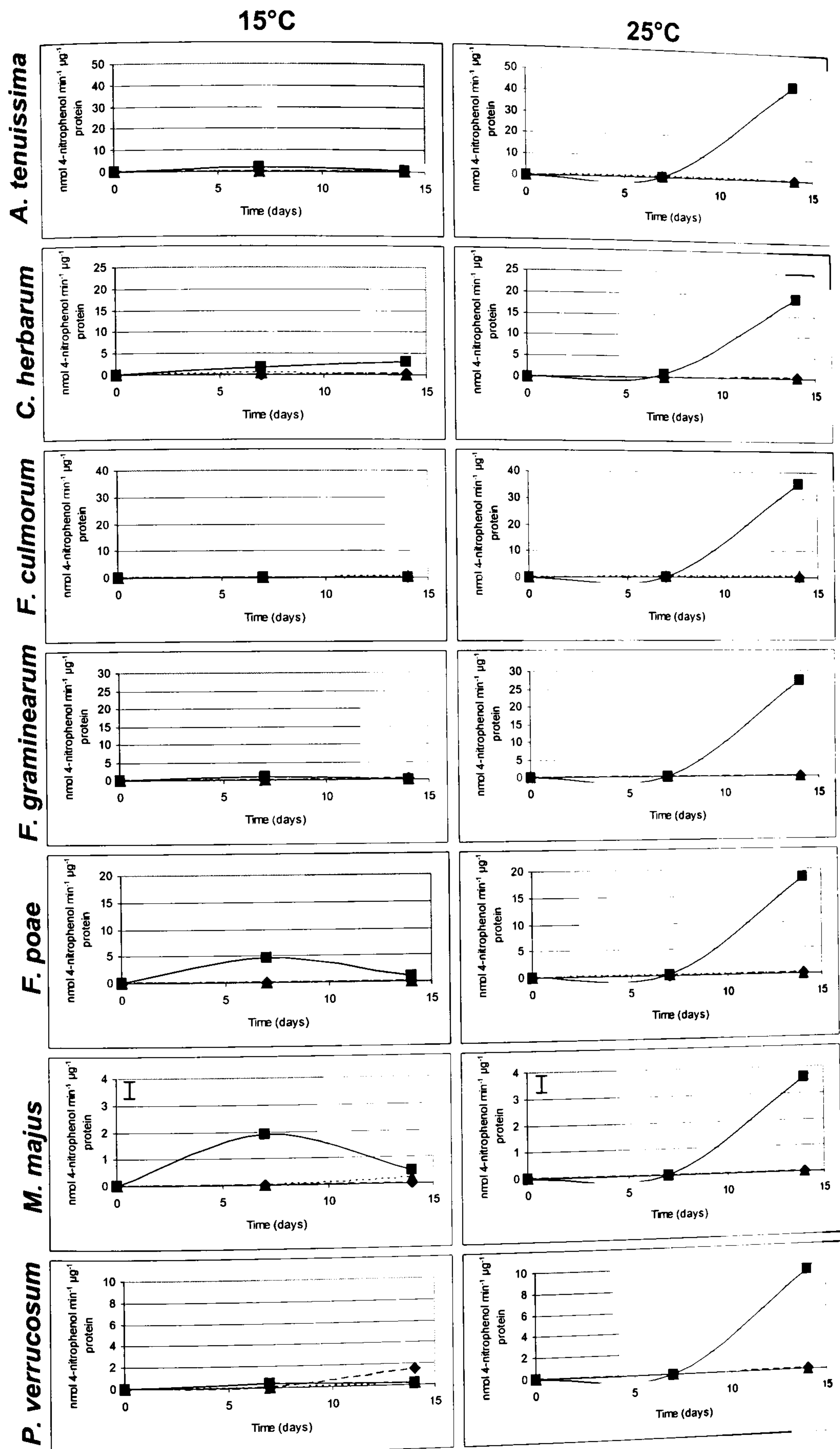


Figure I.13 Specific *N*-acetyl- $\alpha$ -D-glucosaminidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to ▲ 0.99  $a_w$ , ■ 0.95  $a_w$  and ◆ 0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker

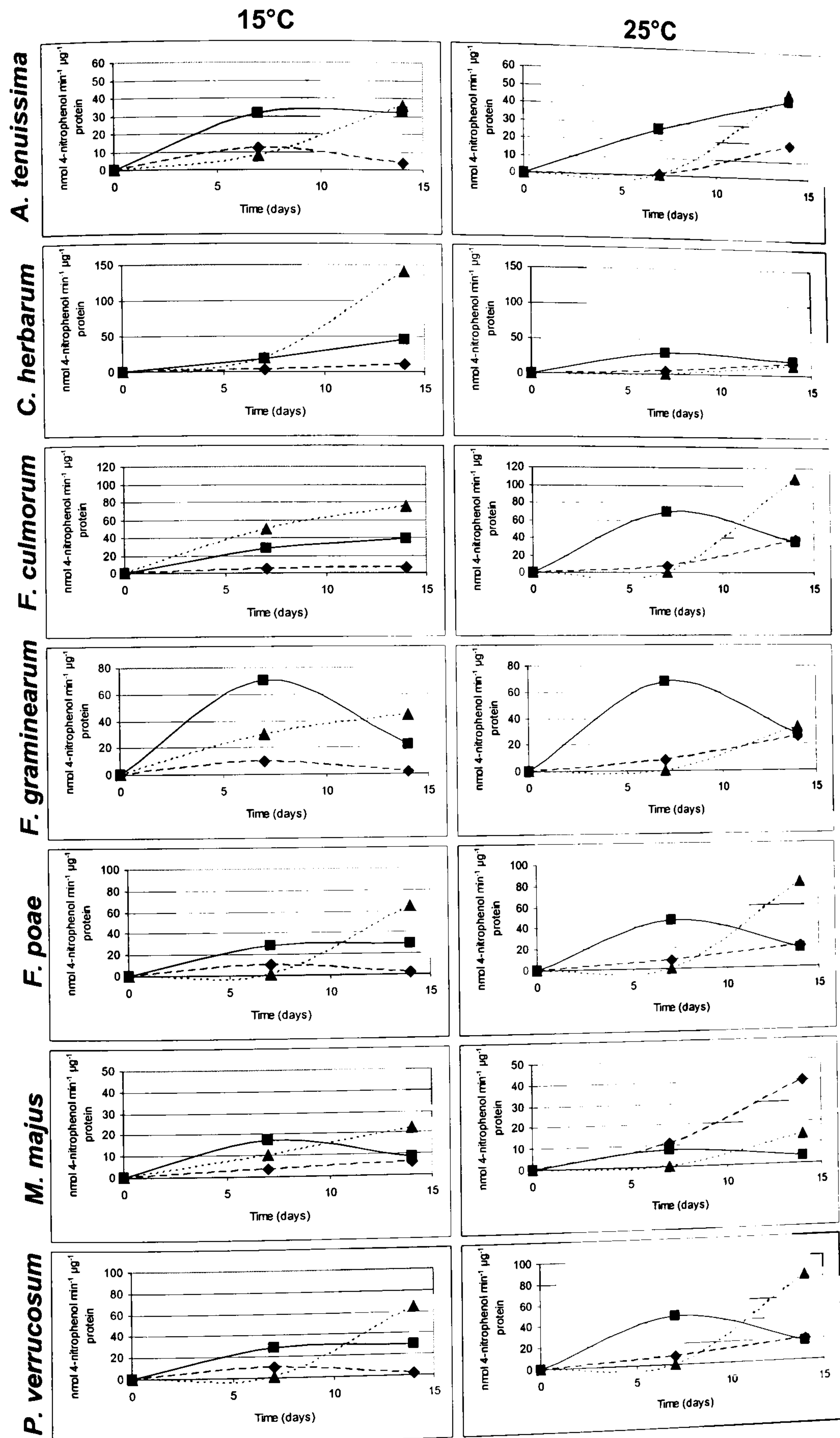


Figure I.14 Specific *N*-acetyl- $\beta$ -D-glucosaminidase activity produced by a range of wheat grain fungi over a 14 day period on irradiated wheat grain adjusted to ▲ 0.99  $a_w$ , ■ 0.95  $a_w$  and ◆ 0.93  $a_w$ . Incubated at 15 °C or 25 °C. LSD shown where larger than marker.



**I.II STATISTICAL ANALYSIS NOT INCLUDED IN MAIN BODY**

**Table I.1** Sources of variation effecting growth of different *F. culmorum* isolates from the north of Europe.

Source	DF	SS	MS	F	P
Isolate	7	43.477	6.211	35.48	<0.001
a <sub>w</sub>	3	1526.005	508.668	2905.87	<0.001
temp	1	251.456	251.456	1436.5	<0.001
Isolate *a <sub>w</sub>	21	120.676	5.746	32.83	<0.001
Isolate *temp	7	23.006	3.287	18.78	<0.001
a <sub>w</sub> *temp	3	178.063	59.354	339.07	<0.001
Isolate* a <sub>w</sub> *temp	21	44.268	2.108	12.04	<0.001
Error	128	22.406	0.175		
Total	191	2209.357			

**Table I.2** Sources of variation effecting fungicide efficacy for control of *F. culmorum* growth on wheat agar.

Source	DF	SS	MS	F	P
Temp	1	55.881	55.881	3443.7	<0.001
Fungicide type	2	247.695	123.848	7632.13	<0.001
a <sub>w</sub>	2	403.158	201.579	1.20E+04	<0.001
Conc	4	321.463	80.366	4952.55	<0.001
Temp* Fungicide type	2	12.422	6.211	382.76	<0.001
Temp* a <sub>w</sub>	2	57.53	28.765	1772.65	<0.001
Temp*Conc	4	13.298	3.324	204.87	<0.001
Fungicide type*a <sub>w</sub>	4	134.343	33.586	2069.73	<0.001
Fungicide type*Conc	8	85.148	10.643	655.9	<0.001
a <sub>w</sub> *Conc	8	94.783	11.848	730.13	<0.001
Temp* Fungicide type *a <sub>w</sub>	4	17.348	4.337	267.27	<0.001
Temp* Fungicide type *Conc	8	3.864	0.483	29.77	<0.001
Temp*a <sub>w</sub> *Conc	8	15.493	1.937	119.34	<0.001
Fungicide type *a <sub>w</sub> *Conc	16	46.743	2.921	180.03	<0.001
Temp* Fungicide type *a <sub>w</sub> *Conc	16	5.426	0.339	20.9	<0.001
Error	180	2.921	0.016		
Total	269	1517.517			



**Table I.3** Sources of variation effecting *F. culmorum* growth when interacting with other species on irradiated wheat grain.

Source	DF	SS	MS	F	P
Interaction	7	31.4786	4.4969	69.6	<0.001
Temp	1	13.2839	13.2839	205.61	<0.001
aw	1	14.9008	14.9008	230.63	<0.001
Interaction*Temp	7	37.883	5.4119	83.76	<0.001
Interaction *aw	7	35.7612	5.1087	79.07	<0.001
Temp*aw	1	23.7533	23.7533	367.65	<0.001
Interaction*Temp*aw	7	26.4851	3.7836	58.56	<0.001
Error	64	4.1349	0.0646		
Total	95	187.6808			

**Table I.4** Sources of variation effecting *F. culmorum* growth when interacting with other species on irradiated wheat grain treated with sub-optimal concentrations of common wheat grain fungicides.

Source	DF	SS	MS	F	P
Interaction	7	59.035	8.434	88.99	<0.001
Fungicide	3	6.812	2.271	23.96	<0.001
Temperature	1	82.896	82.896	874.66	<0.001
a <sub>w</sub>	1	205.319	205.319	2166.39	<0.001
Interact*Fungicide	21	94.356	4.493	47.41	<0.001
Interact*Temp	7	202.332	28.905	304.98	<0.001
Interact*a <sub>w</sub>	7	129.521	18.503	195.23	<0.001
Fungicide*Temp	3	3.407	1.136	11.98	<0.001
Fungicide*a <sub>w</sub>	3	14.618	4.873	51.41	<0.001
Temp*a <sub>w</sub>	1	201.04	201.04	2121.23	<0.001
Interact*Fungicide*Temp	21	69.057	3.288	34.7	<0.001
Interact*Fungicide*a <sub>w</sub>	21	70.05	3.336	35.2	<0.001
Interact*Temp*a <sub>w</sub>	7	62.646	8.949	94.43	<0.001
Fungicide*Temp*a <sub>w</sub>	3	8.452	2.817	29.73	<0.001
Interact*Fungicide*Temp*a <sub>w</sub>	21	78.374	3.732	39.38	<0.001
Error	256	24.262	0.095		
Total	383	1312.175			

**Table I.5** Sources of variation effecting DON levels in the interspecific interaction study on wheat agar.

Source	DF	SS	MS	F	P
Interaction	7	1183.47	169.07	15.58	<0.001
a <sub>w</sub>	2	1029.68	514.84	47.44	<0.001
Fungicide	3	1837.93	612.64	56.45	<0.001
Interaction*a <sub>w</sub>	14	1000.58	71.47	6.59	<0.001
Interaction* Fungicide	21	1529.92	72.85	6.71	<0.001
a <sub>w</sub> * Fungicide	6	1302.42	217.07	20	<0.001
Interact*a <sub>w</sub> * Fungicide	42	1583.73	37.71	3.47	<0.001
Error	192	2083.84	10.85		
Total	287	11551.59			

**Table I.6** Sources of variation effecting NIV levels in the interspecific interaction study on wheat agar.

Source	DF	SS	MS	F	P
Interaction	7	6.5154	0.9308	5.65	<0.001
a <sub>w</sub>	2	12.5238	6.2619	38	<0.001
Fungicide	3	0.5581	0.186	1.13	0.338
Interaction*a <sub>w</sub>	14	12.7498	0.9107	5.53	<0.001
Interaction* Fungicide	21	5.025	0.2393	1.45	0.099
a <sub>w</sub> * Fungicide	6	0.5381	0.0897	0.54	0.774
Interact*a <sub>w</sub> * Fungicide	42	16.0688	0.3826	2.32	<0.001
Error	192	31.6364	0.1648		
Total	287	85.6154			



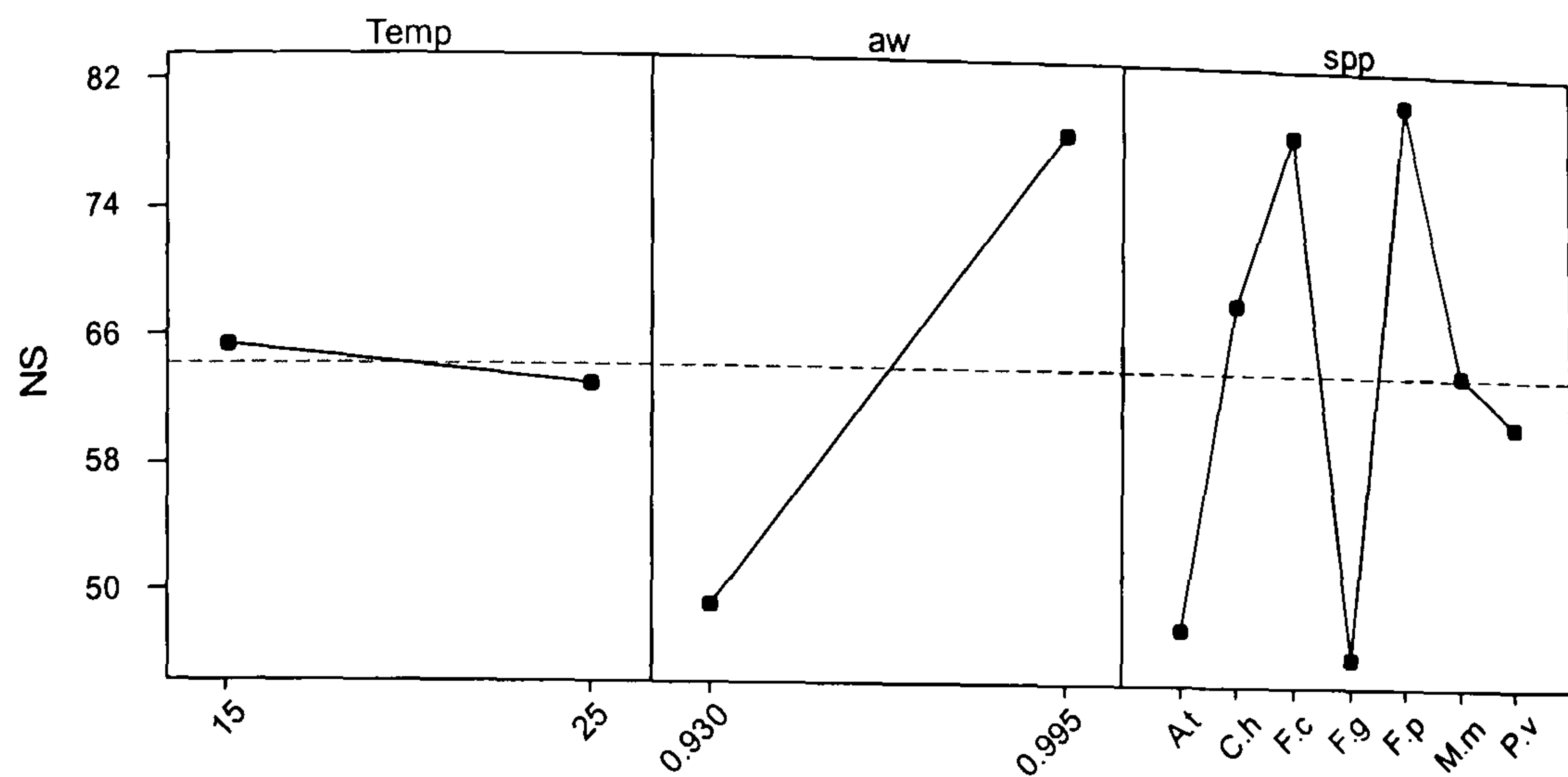
**Table I.7** Sources of variation effecting DON levels in the interspecific interaction study on irradiated wheat grain.

Source	DF	SS	MS	F	P
Interaction	7	28.2615	4.0374	22.25	<0.001
$a_w$	1	6.4577	6.4577	35.59	<0.001
Fungicide	3	27.6383	9.2128	50.77	<0.001
Temp	1	18.24	18.24	100.52	<0.001
Interaction* $a_w$	7	22.3379	3.1911	17.59	<0.001
Interaction*Fungicide	21	26.1966	1.2475	6.87	<0.001
Interaction*Temp	7	22.4413	3.2059	17.67	<0.001
$a_w$ *Fungicide	3	5.9434	1.9811	10.92	<0.001
$a_w$ *Temp	1	8.7667	8.7667	48.31	<0.001
Fungicide*Temp	3	14.3153	4.7718	26.3	<0.001
Interaction* $a_w$ * Fungicide	21	30.2731	1.4416	7.94	<0.001
Interaction* $a_w$ *Temp	7	21.8741	3.1249	17.22	<0.001
Interaction* Fungicide *Temp	21	22.8038	1.0859	5.98	<0.001
$a_w$ *Fungicide *Temp	3	8.2885	2.7628	15.23	<0.001
Interaction* $a_w$ *Fungicide *Temp	21	30.6346	1.4588	8.04	<0.001
Error	256	46.4539	0.1815		
Total	383	340.9268			

**Table I.8** Sources of variation effecting NIV levels in the interspecific interaction study on irradiated wheat grain.

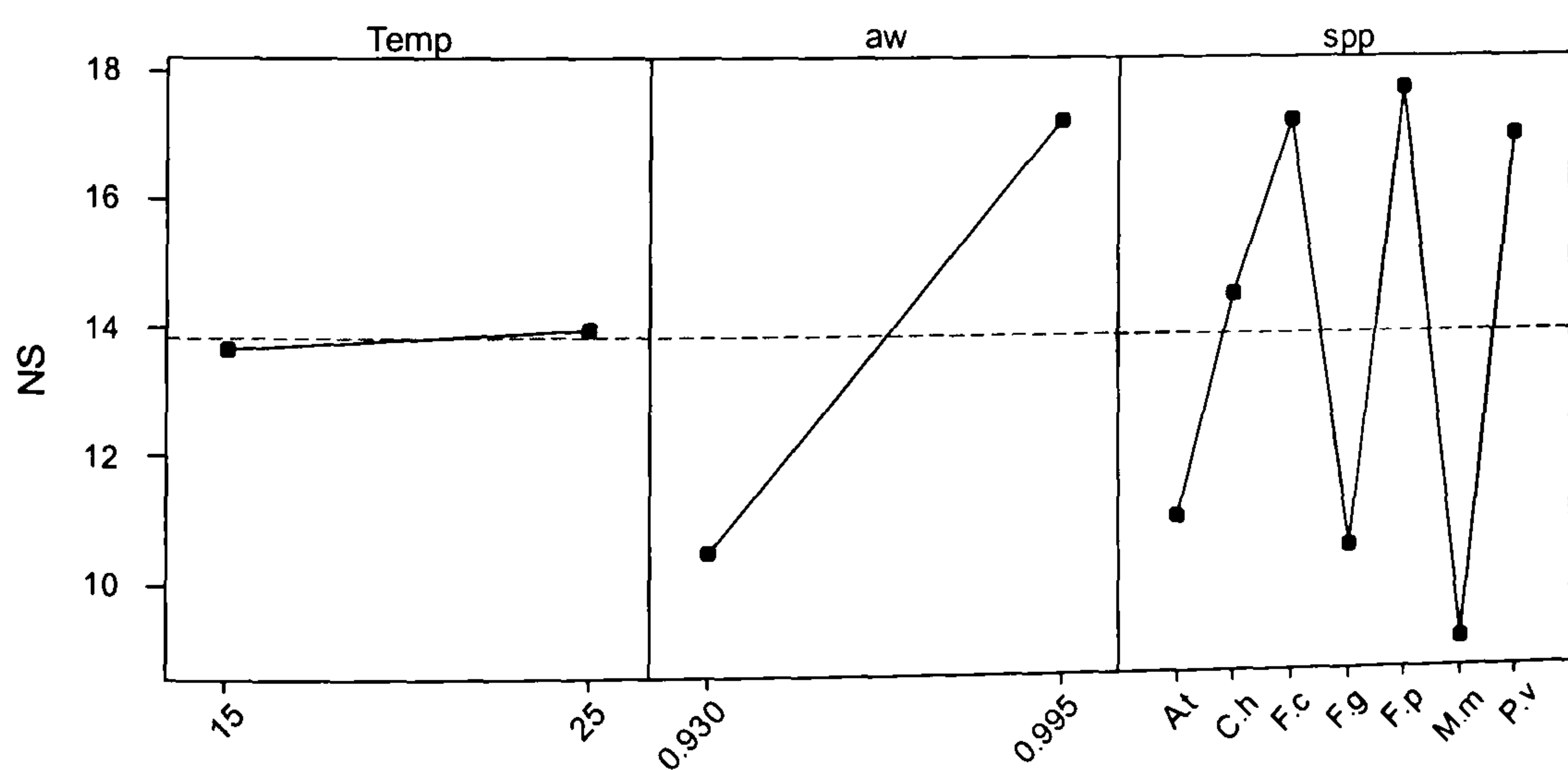
Source	DF	SS	MS	F	P
Interaction	7	0.36361	0.05194	2.53	0.016
$a_w$	1	0.01677	0.01677	0.82	0.367
Fungicide	3	0.17014	0.05671	2.76	0.042
Temp	1	0.00995	0.00995	0.48	0.487
Interaction* $a_w$	7	0.18658	0.02665	1.3	0.251
Interaction*Fungicide	21	1.21422	0.05782	2.82	<0.001
Interaction*Temp	7	0.19224	0.02746	1.34	0.232
$a_w$ *Fungicide	3	0.07799	0.026	1.27	0.286
$a_w$ *Temp	1	0.0513	0.0513	2.5	0.115
Fungicide*Temp	3	0.1856	0.06187	3.02	0.031
Interaction* $a_w$ * Fungicide	21	0.56101	0.02671	1.3	0.173
Interaction* $a_w$ *Temp	7	0.1976	0.02823	1.38	0.216
Interaction* Fungicide *Temp	21	1.25151	0.0596	2.91	<0.001
$a_w$ *Fungicide *Temp	3	0.10866	0.03622	1.77	0.154
Interaction* $a_w$ *Fungicide *Temp	21	0.70537	0.03359	1.64	0.042
Error	256	5.25159	0.02051		
Total	383	10.54415			

## Main Effects Plot - Data Means for NS



**Figure I.15** Main effects of factors effecting niche size when using 94 carbon sources. A.t, *A. tenuissima*, C.h, *C. herbarum*, F.c, *F. culmorum*, F.g, *F. graminearum*, F.p, *F. poae*, M.m, *M. nivale var. majus* and P.v, *P. verrucosum*.

## Main Effects Plot - Data Means for NS



**Figure I.16** Main effects of factors effecting niche size when using 18 key wheat grain carbon sources. A.t, *A. tenuissima*, C.h, *C. herbarum*, F.c, *F. culmorum*, F.g, *F. graminearum*, F.p, *F. poae*, M.m, *M. nivale var. majus* and P.v, *P. verrucosum*.



**Table I.9** Sources of variation for temporal total (TA) and specific (SA) activity of hydrolytic enzymes produced by *A. tenuissima*, *C. herbarum*, *F. culmorum*, *F. graminearum*, *F. poae*, *M. nivale* var. *majus* and *P. verrucosum*.

Source	P TA	P SA
Spp	<0.001	<0.001
Temp	<0.001	0.244
Aw	<0.001	<0.001
Week	<0.001	0.003
Substrat	<0.001	<0.001
Spp*Temp	<0.001	<0.001
Spp*Aw	<0.001	<0.001
Spp*Week	<0.001	<0.001
Spp*Substrat	<0.001	<0.001
Temp*Aw	<0.001	<0.001
Temp*Week	<0.001	<0.001
Temp*Substrat	<0.001	<0.001
Aw*Week	<0.001	<0.001
Aw*Substrat	<0.001	<0.001
Week*Substrat	<0.001	<0.001
Spp*Temp*Aw	<0.001	<0.001
Spp*Temp*Week	<0.001	<0.001
Spp*Temp*Substrat	<0.001	<0.001
Spp*Aw*Week	<0.001	<0.001
Spp*Aw*Substrat	<0.001	<0.001
Spp*Week*Substrat	<0.001	<0.001
Temp*Aw*Week	<0.001	<0.001
Temp*Aw*Substrat	<0.001	<0.001
Temp*Week*Substrat	<0.001	<0.001
Aw*Week*Substrat	<0.001	<0.001
Spp*Temp*Aw*Week	<0.001	<0.001
Spp*Temp*Aw*Substrat	<0.001	<0.001
Spp*Temp*Week*Substrat	<0.001	<0.001
Spp*Aw*Week*Substrat	<0.001	<0.001
Temp*Aw*Week*Substrat	<0.001	<0.001
Spp*Temp*Aw*Week*Substrat	<0.001	<0.001

**Table I.10** Sources of variation for efficacy of essential oils and antioxidants for control of growth of *F. culmorum*, *F. graminearum* and *F. poae* on wheat agar.

Source	DF	SS	MS	F	P
$a_w$	2	560.06	280.03	3257.43	<0.001
Temp	1	888.989	888.989	1.00E+04	<0.001
Spp	2	46.577	23.288	270.9	<0.001
Treatment	4	279.512	69.878	812.85	<0.001
Conc	5	2962.697	592.539	6892.66	<0.001
$a_w$ *Temp	2	133.333	66.666	775.49	<0.001
$a_w$ *Spp	4	20.954	5.238	60.94	<0.001
$a_w$ *Treatment	8	35.483	4.435	51.59	<0.001
$a_w$ *Conc	10	444.849	44.485	517.47	<0.001
Temp* Spp	2	23.063	11.532	134.14	<0.001
Temp*Treatment	4	75.47	18.868	219.48	<0.001
Temp*Conc	5	546.893	109.379	1272.34	<0.001
Species*treatment	8	14.179	1.772	20.62	<0.001
Species*Conc	10	40.672	4.067	47.31	<0.001
Treatment*Conc	20	239.188	11.959	139.12	<0.001
$a_w$ *Temp*Spp	4	73.308	18.327	213.19	<0.001
$a_w$ *Temp*Treatment	8	14.037	1.755	20.41	<0.001
$a_w$ *Temp*Conc	10	85.252	8.525	99.17	<0.001
$a_w$ *Spp*Treatment	16	17.958	1.122	13.06	<0.001
$a_w$ *Spp*conc	20	23.391	1.17	13.6	<0.001
$a_w$ *Treatment*Conc	40	43.202	1.08	12.56	<0.001
Temp*Spp*Treatment	8	14.867	1.858	21.62	<0.001
Temp*Spp*Conc	10	13.379	1.338	15.56	<0.001
Temp*Treatment*Conc	20	108.09	5.404	62.87	<0.001
Spp*Treatment*Conc	40	26.663	0.667	7.75	<0.001
$a_w$ *Temp*Spp*Treatment	16	13.69	0.856	9.95	<0.001
$a_w$ *Temp*Spp*Conc	20	57.188	2.859	33.26	<0.001
$a_w$ *Temp*Treatment*Conc	40	27.113	0.678	7.88	<0.001
$a_w$ *Spp*Treatment*Conc	80	41.032	0.513	5.97	<0.001
Temp*Spp*Treatment*Conc	40	29.855	0.746	8.68	<0.001
$a_w$ *Temp*Spp*Treatment*Conc	80	42.545	0.532	6.19	<0.001
Error	540	46.422	0.086		
Total	1079	6989.91			



**Table I.11** Sources of variation in DON, NIV, HT2 and T2 produced by *F. culmorum*, *F. graminearum* and *F. poae* on irradiated wheat grain treated with essential oils or antioxidants (Conc 0 - 500  $\mu\text{g g}^{-1}$ ) at 15 – 25 °C and 0.95 – 0.995  $a_w$ .

Source	P (DON)	P (NIV)	P (HT2)	P (T2)
Spp	<0.001	<0.001	<0.001	<0.001
Temp	<0.001	<0.001	<0.001	<0.001
aw	<0.001	<0.001	<0.001	<0.001
Treatmen	<0.001	<0.001	<0.001	<0.001
Conc	<0.001	<0.001	<0.001	<0.001
Spp*Temp	<0.001	<0.001	<0.001	<0.001
Spp*aw	<0.001	<0.001	<0.001	<0.001
Spp*Treatmen	<0.001	<0.001	<0.001	<0.001
Spp*Conc	<0.001	<0.001	<0.001	<0.001
Temp*aw	<0.001	<0.001	<0.001	<0.001
Temp*Treatmen	<0.001	<0.001	<0.001	<0.001
Temp*Conc	<0.001	<0.001	<0.001	<0.001
aw*Treatmen	<0.001	<0.001	<0.001	<0.001
aw*Conc	<0.001	<0.001	<0.001	0.126
Treatmen*Conc	<0.001	<0.001	<0.001	<0.001
Spp*Temp*aw	<0.001	<0.001	<0.001	<0.001
Spp*Temp*Treatmen	<0.001	<0.001	<0.001	<0.001
Spp*Temp*Conc	<0.001	<0.001	<0.001	<0.001
Spp*aw*Treatmen	<0.001	<0.001	<0.001	<0.001
Spp*aw*Conc	<0.001	<0.001	<0.001	0.096
Spp*Treatmen*Conc	<0.001	<0.001	<0.001	<0.001
Temp*aw*Treatmen	<0.001	<0.001	<0.001	<0.001
Temp*aw*Conc	<0.001	<0.001	0.029	<0.001
Temp*Treatmen*Conc	<0.001	<0.001	<0.001	<0.001
aw*Treatmen*Conc	<0.001	<0.001	<0.001	<0.001
Spp*Temp*aw*Treatmen	<0.001	<0.001	<0.001	<0.001
Spp*Temp*aw*Conc	<0.001	<0.001	0.009	<0.001
Spp*Temp*Treatmen*Conc	<0.001	<0.001	<0.001	<0.001
Spp*aw*Treatmen*Conc	<0.001	<0.001	<0.001	<0.001
Temp*aw*Treatmen*Conc	<0.001	<0.001	<0.001	<0.001
Spp*Temp*aw*Treatmen*Conc	<0.001	<0.001	<0.001	<0.001

**Table I.12** Sources of variation for total fungal population (CFUs) in naturally contaminated wheat grain treated with 200  $\mu\text{g g}^{-1}$  of bay, clove and cinnamon essential oils and BHA and propyl paraben antioxidants.

Source	DF	SS	MS	F	P
Aw	2	2.10E+14	1.05E+14	15.48	<0.001
Temp	1	4.58E+14	4.58E+14	67.57	<0.001
Time	3	9.56E+14	3.19E+14	47	<0.001
Treat	5	6.95E+14	1.39E+14	20.49	<0.001
Aw*Temp	2	1.21E+14	6.04E+13	8.9	<0.001
Aw*Time	6	1.57E+14	2.62E+13	3.86	0.001
Aw*Treat	10	2.04E+14	2.04E+13	3.01	0.001
Temp*Time	3	8.08E+14	2.69E+14	39.72	<0.001
Temp*Treat	5	4.78E+14	9.57E+13	14.11	<0.001
Time*Treat	15	1.37E+15	9.11E+13	13.44	<0.001
Aw*Temp*Time	6	1.43E+14	2.38E+13	3.51	0.002
Aw*Temp*Treat	10	1.19E+14	1.19E+13	1.75	0.069
Aw*Time*Treat	30	3.59E+14	1.20E+13	1.76	0.01
Temp*Time*Treat	15	1.05E+15	6.99E+13	10.3	<0.001
Aw*Temp*Time*Treat	30	5.45E+14	1.82E+13	2.68	<0.001
Error	288	1.95E+15	6.78E+12		
Total	431	9.62E+15			

**Table I.13** Sources of variation for DON levels in naturally contaminated wheat grain treated with 200  $\mu\text{g g}^{-1}$  of bay, clove and cinnamon essential oils and BHA and propyl paraben antioxidants after 28 days of incubation.

Source	DF	SS	MS	F	P
aw	2	118.138	59.069	23.92	<0.001
temp	1	63.494	63.494	25.71	<0.001
Treatmen	5	51.266	10.253	4.15	0.002
aw*temp	2	118.138	59.069	23.92	<0.001
aw*Treatmen	10	99.985	9.999	4.05	<0.001
temp*Treatmen	5	51.266	10.253	4.15	0.002
aw*temp*Treatmen	10	99.985	9.999	4.05	<0.001
Error	72	177.815	2.47		
Total	107	780.086			



# APPENDIX II

## II.I PEER REVIEWED PUBLICATIONS

**Hope, R. & Magan, N. (2003).** Multi-target environmental approach for control of growth and toxin production by *Fusarium culmorum* using essential oils and antioxidants. In *Advances in Stored Product Protection*. Eds. P.Credland. Cabi Publishing. In Press.

**Magan, N., Hope, R., Cairns, V. & Aldred, D. (2003).** Post-harvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. *European Journal of Plant Pathology*. In Press.

**Hope, R. & Magan, N. (2003)** Two-dimensional environmental profiles of growth, deoxynivalenol and nivalenol production by *Fusarium culmorum* on a wheat-based substrate. *Letters in Applied Microbiology* **37**. 70-74.

**Magan, N., Hope, R., Colleate, A. & Baxter, E.S. (2002).** Relationship between growth and mycotoxin production by *Fusarium species*, biocides and environment. *European Journal of Plant Pathology* **108**, 685-690.

**Hope, R., Colleate, A., Baxter, E. S., & Magan, N. (2000)** Interactions between environmental stress and fungicides affect growth and mycotoxin production by *Fusarium culmorum* isolates from wheat grain. The BCPC Conference - Pests & Diseases 2000 **3**. 889-894.



## II.II POSTER PRESENTATIONS

**Hope, R. & Magan, N. (2003).** Ecophysiology of growth and deoxynivalenol/nivalenol production by *F. culmorum* and *F. graminearum*. 9<sup>th</sup> International Fusarium Workshop, Sydney, Australia. In Press.

**Hope, R. & Magan, N. (2002).** A multitarget environmental approach to control of growth and mycotoxin production by *Fusarium* spp. using essential oils and antioxidants. 7<sup>th</sup> International Mycological Congress, Oslo, Norway. Abstract p. 250.

**Hope, R., Colleate, A., Baxter, E. S., & Magan, N. (2000)** Interactions between environmental stress and fungicides affect growth and mycotoxin production by *Fusarium culmorum* isolates from wheat grain. The BCPC Conference - Pests & Diseases 2000

The following papers are being finalised and will be submitted for review:-

**Hope, R., Aldred, D. & Magan, N. (2003).** Comparison of environmental profiles for growth and deoxynivalenol production by *Fusarium culmorum* and *F. graminearum* on wheat grain.

**Hope, R., Jestoi, M. & Magan, N. (2003).** Efficacy of essential oils and antioxidants for control of growth and mycotoxin production by *Fusarium culmorum* and *Fusarium graminearum* under different environmental conditions